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ENDOTHELIAL NOTCH LIGANDS IN BONE MARROW FUNCTION AND IN MALIGNANCY

Inês Sofia Alvarez Martins

Orientador:
Professor Doutor Sérgio Jerónimo Rodrigues Dias

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas, especialidade de Biologia Celular e Molecular

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Fundação para a Ciência e Tecnologia, bolsa SFRH/BD/78249/2011

2017

A impressão desta tese foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 19 de Abril de 2016

ACKNOWLEDGMENTS

Though only my name appears on the cover of this dissertation, a great many people have contributed to its production. I owe my debt of gratitude to all those who made this degree possible, for their scientific and, mostly, moral support throughout these challenging few years.

First of all, I would like to thank my supervisor, Dr. Sérgio Dias, for his support and contributions throughout this process. His patience and encouragement have helped me overcome many setbacks and his mentorship was essential for my success.

The people I've crossed paths with during this journey have made this experience one that I will cherish forever. None of this would have been accomplished without the past and present members of the SDias lab. I would like to thank Leonor, for her teaching when I first came to the lab, Ana Magalhães, Sandrina and Hélia, for the scientific discussions, and especially to Ana de Barros, Inês Matias, Clemente and Cabrita for putting up with my morning grumpiness, for the joyful environment in the lab, and for the great friendship. Also, to the iMMers who contributed for this work, especially to the members of the histology, flow cytometry, imaging and rodent facilities, for all the advices and technical support.

I am grateful that I have been surrounded by good friends who have kept me sane and my views in perspective in the process. A heartfelt thank you goes particularly to Isaura, Diogo, Memé, Marta, Alice and Joana who have been the most wonderful supporters in the quest for my PhD.

Most importantly, none of this would have been possible without the love and patience of my family. My wonderful parents, who never stopped trying to understand just what I was doing and who have been a constant source of strength and encouragement all these years. My little brother, Rui, who is the most amazing enthusiast in live. When my confidence wavered, you inspired me. I am deeply grateful.

RESUMO

O sistema cardiovascular é o primeiro a ser formado no embrião de organismos vertebrados, sendo composto por uma rede de vasos que permite a circulação do sangue por todo o corpo, entregando oxigénio e nutrientes. As células endoteliais que formam os vasos sanguíneos têm um papel instrutor em diversos processos fisiológicos e patológicos, como a manutenção das células estaminais, a regeneração e reparação de órgãos ou o desenvolvimento de tumores, através da produção e libertação de factores parácrinos, designados factores angiócrinos. Por conseguinte, a fim de compreender de que forma as células endoteliais comunicam com as células circundantes, a identificação destes factores tem recebido muita atenção nos últimos anos.

Nesta Tese usámos ratinhos geneticamente modificados para explorar o envolvimento de dois factores angiócrinos pertencentes à via de sinalização Notch - Jagged 1 (Jag1) e Delta-like 4 (Dll4) - em dois processos distintos. Primeiro explorámos a função do ligando Jag1 especificamente em células endoteliais no recrutamento de macrófagos para tumores da próstata em desenvolvimento. Com esse intuito, bloqueámos Jag1 especificamente em células endoteliais VE-Caderina^{+} e sobrexpressámos Jag1 em células endoteliais positivas para Tie2, em ratinhos TRAMP*Jag1$^{lox/lox}$*VE-Cadherin-Cre-ERT2 (eJag1^{KO}) e TRAMP*Tet-O-Jag1*Tie2-rtTA (eJag1^{OE}), respectivamente. Em ratinhos que desenvolvem tumores da próstata espontâneos (TRAMP), a expressão do ligando Jag1 em células endoteliais induz a proliferação de células tumorais, promove angiogénese e maturação dos novos vasos. No entanto, o envolvimento de Jag1 endotelial (eJag1) no recrutamento e activação de macrófagos para o tumor ainda não era conhecida. O segundo processo que estudámos foi a modulação da hematopoiese e do nicho vascular da medula óssea através da modulação de Dll4 endotelial, usando ratinhos com mutações condicionais e indutíveis que promovem o knock-out ou a sobrexpressão de Dll4 em células endoteliais. Um vasto número de estudos mostrou o envolvimento de Dll4 no desenvolvimento vascular e na diferenciação de células hematopoiéticas, mas o papel que o ligando desempenha no nicho vascular e na comunicação das células endoteliais com células hematopoiéticas na medula óssea não tinha sido estudado.

O primeiro objectivo desta tese foi investigar se o ligando Jag1 em células endoteliais poderia afectar o desenvolvimento de tumores da próstata através do recrutamento de macrófagos para o tumor. Os macrófagos são um grande componente celular do microambiente tumoral e podem ser activados diferencialmente para um fenótipo anti- (M1) ou pro-tumoral (M2). As células endoteliais e os macrófagos estabelecem interacções específicas que modulam as propriedades angiogénicas do tumor e que se pensa serem capazes de afectar a polarização dos macrófagos. Os nossos resultados mostram que eJag1 regula positivamente o recrutamento e polarização de macrófagos para um fenótipo pro-tumoral em tumores da próstata. De facto, ratinhos eJag1^{KO} têm menos macrófagos intra-tumorais que os controlos e maior polarização M1 e ratinhos eJag1^{OE} mais macrófagos que os respectivos controlos, e maior polarização M2. Este fenótipo foi também observado *in vitro* quando células endoteliais do cordão umbilical (HUVECs) tratadas com um anticorpo neutralizante para Jag1 promoveram uma diminuição do número de macrófagos do tipo M2, sugerindo que esta modulação da activação de macrófagos é um efeito directo da expressão de Jag1 em células endoteliais.

Investigámos a modulação da expressão de genes que codificam para factores angiócrinos e demonstrámos que a modulação dos níveis de Jag1 no endotélio afectou a expressão de moléculas de adesão e de quimioatractores, bem como de outros ligandos da via Notch. Particularmente em ratinhos eJag1^{OE}, a sobrexpressão de Jag1 induziu um aumento nos níveis de *Angpt2*, *Dll4* e *Jag2* e uma subexpressão de *Cxcl12*, *Vcam1* e *Dll1* nas células endoteliais isoladas do tumor. O aumento de Angpt2, em particular, pode explicar o recrutamento e polarização dos macrófagos para um fenótipo M2.

De forma semelhante, os macrófagos M1 e M2 também mostraram alterações na expressão de alguns genes. Os níveis de eJag1 correlacionavam-se com um aumento da expressão de *Il6* e *Tnfa* e com a subexpressão de *Notch2*, particularmente em macrófagos M2. Adicionalmente, macrófagos M2 também exibiam uma sobrexpressão de *Notch1* e *Cxcr4*, independentemente da modulação de eJag1. Em conclusão, a expressão de Jag1 em células endoteliais promove o recrutamento de macrófagos para tumores da próstata e polarização para um fenótipo M2, possivelmente através da indução da modificação da expressão de outros factores angiócrinos o que induz uma modulação nos padrões de expressão genética dos macrófagos.

A fim de compreender de que forma a expressão do ligando Dll4 no nicho vascular afectava a hematopoiese, tirámos partido de ratinhos com ganho e perda-de-função de Dll4 especificamente em células endoteliais (eDll4^{OE} e eDll4^{KO}) e analisámo-los sem serem irradiados ou 8 e 26 dias após serem submetidos a uma irradiação sub-letal, que induziu mielossupressão. Em animais não irradiados, a modulação de Dll4 endotelial (eDll4) perturbou a hematopoiese, como evidenciado pela redução de células mielóides (CD11b^{+}) no sangue periférico de ratinhos eDll4^{OE} e pelo aumento de células linfóides B (B220^{+}) na medula óssea e sangue periférico de ratinhos eDll4^{KO}. Adicionalmente, ensaios de diferenciação em metilcelulose *in vitro* demonstraram que ratinhos knock-out para Dll4 endotelial têm um aumento de progenitores mielóides (CFU-G) mas uma diminuição no número de progenitores multipotentes (CFU-GEMM).

Após exposição a radiação, ratinhos com menores níveis de eDll4 (eDll4^{KO} e Control OE) recuperaram mais rapidamente o número de células na medula que os restantes modelos. Detectámos também uma diminuição no número de plaquetas em ratinhos eDll4^{OE} e um aumento de eritrócitos em circulação em ratinhos eDll4^{KO}, 8 dias após mielossupressão. Vinte e seis dias após irradiação, as proporções relativas das diferentes linhagens hematopoiéticas também tinham sido modificadas em função dos níveis de eDll4. A sobrexpressão de Dll4 induziu diferenciação mielóide e linfóide T, em detrimento da linhagem linfóide B, e o oposto foi detectado em ratinhos eDll4^{KO}, consistente com o papel atribuído ao ligando Dll4 na especificação das linhagens hematopoiéticas, quando expresso noutro tipo de células do microambiente. Analisámos ainda a recuperação hematopoiética após mielossupressão no contexto de transplante de medula. Transplante de medula total de ratinhos eDll4^{KO} para ratinhos controlo letalmente irradiados promoveu uma recuperação da celularidade da medula mais rápida, e diminuiu o dano na medula óssea, quando comparado com um transplante de medula de ratinhos controlo para ratinhos eDll4^{KO}.

Esta modificação no compartimento hematopoiético levou-nos a questionar se o nicho vascular da medula óssea também seria modificado pela modulação de eDll4. Verificámos que o nicho vascular não estava alterado em ratinhos que não tinham sido expostos a radiação ou que foram sacrificados 26 dias após mielossupressão, mas que 8 dias após

irradiação, ratinhos eDll4^{KO} tinham um aumento de vasos positivos para VE-Caderina e VEGFR2 e ratinhos eDll4^{OE} exibiam uma diminuição de vasos VEGFR2-positivos. Esta modulação da identidade dos vasos na medula óssea, sem afectar o número total de vasos (CD105^{+}), pode explicar a recuperação mais rápida de ratinhos eDll4^{KO} quando expostos a radiação. Adicionalmente, tal modulação foi acompanhada de uma modificação na localização de células B e de megacariócitos relativamente aos sinusoides da medula óssea. Oito dias após mielossupressão, ratinhos com menores níveis de eDll4 tinham menos células B e megacariócitos em contacto com vasos VE-Caderina^{+}, o que se correlacionou com menos células B dentro dos vasos ou em circulação 26 dias após irradiação. Investigámos ainda a modulação da expressão de genes que codificam factores angiócrinos que poderiam modular a hematopoiese. A sobrexpressão de eDll4 induziu uma diminuição da expressão de *Cxcl12*, *Vcam1* e *Thpo* e o knock-out de eDll4 levou a uma subexpressão de *Cxcl12*. Particularmente em ratinhos eDll4^{KO}, a diminuição do número de células B e megacariócitos em contacto com os sinusoides pode ser explicada pela redução dos níveis de *Cxcl12*, uma vez que o tratamento de HUVECs com um anticorpo neutralizante para Dll4 levou a uma redução dos níveis de *CXCL12* e uma consequente diminuição na migração de células CD34^{+}. A diminuição do número de plaquetas em ratinhos eDll4^{OE}, por sua vez, pode ser explicado pela redução dos níveis de *Thpo*.

O trabalho realizado neste projecto de doutoramento revelou os efeitos da modulação de eJag1 e eDll4 na progressão de tumores da próstata e na recuperação da medula óssea após mielossupressão, respectivamente. Em ambos os casos, a expressão dos ligandos da via Notch em células endoteliais revelou ser prejudicial. eJag1 promove directa e indirectamente a progressão de tumores e eDll4 bloqueia a normal recuperação da medula após irradiação sub-letal ou num cenário de transplante de medula, indicando que estes ligandos devem ser considerados como possíveis alvos terapêuticos.

Palavras-chave

Tumor da próstata; Jagged 1; Macrófagos; Polarização de macrófagos; M1; M2; Medula Óssea; Nicho vascular; Delta-like 4; Hematopoiese; Sinalização Notch

SUMMARY

Endothelial cells have emerged as instructive players in distinct physiological and pathological tasks, maintaining resident stem cell homeostasis, orchestrating tissue regeneration, and inducing tumor growth through the release of paracrine factors, known as angiocrine factors. In this Thesis, we explored the role of two particular angiocrine genes, Jagged 1 (Jag1) and Delta-like 4 (Dll4), in the development of prostate tumors through the recruitment of macrophages and in bone marrow (BM) regeneration following myeloablation, respectively.

To address endothelial Jag1 (eJag1) function in prostate tumor progression, we used genetically engineered mouse models, in which mice that develop spontaneous prostate tumors (TRAMP) were crossed with endothelial-specific Jag1 loss- or gain-of-function mice (eJag1^{KO} and eJag1^{OE}). We showed that eJag1 induces macrophage recruitment into the tumors and polarization into a pro-tumoral M2 phenotype, both *in vivo* and *in vitro*. This was accompanied by a modulation in angiocrine gene expression, particularly in eJag1^{OE} mice, and in the macrophage expression pattern. Our preliminary data thus suggest that eJag1 modulates tumor growth and angiogenesis indirectly by promoting macrophage recruitment and polarization into an M2 state.

To understand how endothelial Dll4 modulation affected the BM vascular niche and hematopoiesis, we used two conditional mouse models with endothelial-specific Delta-like 4 (Dll4) loss- or gain-of-function (eDll4^{KO} and eDll4^{OE}) and analyzed their hematopoietic and vascular compartments with and without sub-lethal irradiation. Although the BM vascular niche was not affected by eDll4 modulation at steady state or 26 days after irradiation, by day 8 post-irradiation eDll4 induced changes in BM vessel identity, without affecting the overall BM vessel content. This modulation of the BM vascular niche was accompanied by a modulation in the angiocrine gene expression pattern and induced changes in hematopoiesis. Particularly, eDll4 levels correlated with increased erythropoiesis and decreased megakaryopoiesis and B lymphopoiesis, although it promoted the migration of B cells and megakaryocytes to the vicinity of BM sinusoids. These modifications resulted in an improvement of hematopoietic recovery both in sub-lethally irradiated eDll4^{KO} and in lethally irradiated control mice transplanted with the BM of eDll4^{KO} mice, suggesting that

eDll4 impairs BM recovery following myeloablation and may be relevant in a setting of BM transplantation or in patients receiving chemotherapy.

Taken together, our data suggests that the Notch ligands Jag1 and Dll4 may be possible targets in tumor progression and BM recovery, respectively, as their expression in endothelial cells is unfavorable in a clinical setting.

Keywords

Prostate tumor; Jagged 1; Macrophage; Macrophage polarization; M1; M2; Bone marrow; Vascular niche; Delta-like 4; Hematopoiesis; Notch signaling

ABBREVIATIONS

ADAM	A desintegrin and metalloprotease
Angpt	Angiopoietin
BC	Before Christ
BM	Bone marrow
CAR	CXCL12 abundant reticular cells
CD	Cluster of differentiation
CDC42	Cell division cycle 42
CFU	Colony forming unit
CFU-E	CFU-erythrocyte
CFU-G	CFU-granulocyte
CFU-GEEM	CFU-granulocyte-erythrocyte-macrophage-megakaryocyte
CFU-M	CFU-macrophage
CLP	Common lymphoid progenitor
COUP-TFII	Chicken ovalbumin upstream promoter transcription factor II
Cox	Cyclooxygenase
CSF	Colony-stimulating factor
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
Dll	Delta-like
E	Embryonic day
EC	Endothelial cell
ECM	Extracellular matrix
eDll4	Endothelial Dll4
eDll4^{KO}	Endothelial Dll4 knockout mice (Dll4$^{lox/lox}$*VE-Cadherin-Cre-ERT2)
eDll4^{OE}	Endothelial Dll4 overexpressing mice (Dll4-Tet-O7*Tie2-rtTA)
EGF	Epidermal growth factor
eJag1	Endothelial Jag1
eJag1^{KO}	Endothelial Jag1 knockout mice (TRAMP*Jag1$^{lox/lox}$*VE-Cadherin-Cre-ERT2)
eJag1^{OE}	Endothelial Jag1 overexpressing mice (TRAMP*Tet-O-Jag1*Tie2-rtTA)
EPC	Endothelial progenitor cell
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FBS	Fetal bovine serum
FGF	Fibroblast growth factor

FGFR	Fibroblast growth factor receptor
G-CSF	Granulocyte colony-stimulating factor
GPCR	G-protein-coupled receptor
GTPase	Guanosine triphosphatase
HES	Hairy/enhancer of split
HEY	HES-related protein
HIF	Hypoxia inducible factor
HUVEC	Human umbilical cord vein endothelial cells
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
ICAM1	Intercellular adhesion molecule 1
IFNγ	Interferon gamma
IGF	Insulin-like growth factor
IL	Interleukin
IP	Intraperitoneally
Jag	Jagged
JAM	Junctional adhesion molecules
LepR	Leptin receptor
LPS	Lipopolysaccharide
LSEC	Liver sinusoidal endothelial cells
LT-HSC	Long term hematopoietic stem cell
Maml	Mastermind-like
MAPK	Mitogen-activated protein kinase
MCP1	Monocyte chemoattractant protein 1
MHC II	Major histocompatibility complex class II
MK	Megakaryocyte
MMP	Matrix metalloprotease
MSC	Mesenchymal stromal cell
MT1-MMP	Membrane-type 1 matrix metalloprotease
N-Cadherin	Neuronal cadherin
Nes	Nestin
NFκB	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NICD	Notch intracellular domain
NO	Nitric oxide
NOS3	Nitric oxide synthase 3
Nrp	Neuropilin

Osx	Osterix
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
PlGF	Placental growth factor
Pre-pro-B	Early B lymphocyte progenitor
Pre-B	Late B lymphocyte progenitor
Prx1	Paired related homeobox protein 1
RBPjk	Recombining binding protein suppressor of hairless kappa
Robo4	Roundabout 4
Sca1	Stem cell antigen 1
SCF	Stem cell factor
SDF1	Stromal derived factor 1
SEC	Sinusoidal endothelial cell
SMA	Smooth muscle actin
SMC	Smooth muscle cell
TAMs	Tumor-associated macrophages
TEM	Tie2-expressing monocyte/macrophage
TGF	Transforming growth factor
Thpo	Thrombopoietin
Tie	Tyrosine kinase with immunoglobulin-like EGF-like domains
TNFα	Tumor necrosis factor
TRAMP	Transgenic adenocarcinoma mouse prostate
Unc5b	Unc-5 homolog b
VCAM1	Vascular cell adhesion molecule 1
VE-Cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VHL	von Hippel-Lindau protein
vWF	Von Willebrand Factor
ZO	Zona occludens

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INTRODUCTION

CONTENTS

1.1. THE VASCULAR SYSTEM

The human body can be described as a series of biological systems that function together to sustain life, each serving a particular function. One of these systems, the circulatory or cardiovascular system, is comprised of a complex network of hollow tubes that allow blood to circulate throughout the entire body, delivering oxygen and nutrients to all tissues. One of the earliest and most accurate descriptions of the cardiovascular system was made by Aristoteles in 350 BC, who placed the heart at the center of the vascular system and understood that both arteries and veins originated from it (Praagh & Praagh 1983; Shoja et al. 2008). Later, in the second century, Galen demonstrated that arteries, and not just veins, contained blood and not air and verified that the systems of arteries and veins were completely distinct, differing in their location, their capacity of pulsating, the thickness of their tunic and the type of blood they carried (Aird 2011; Khan et al. 2005). However, he wrongly believed that the arterial and venous systems were closed and separated, communicating only through extremely small and invisible pores in the septum that separated both ventricles. In his model, the blood was not recycled but constantly formed in the liver from the ingested food and then consumed by the organs. It was only in the 17th century that William Harvey postulated that the blood flows through the body in a circular motion, pumped by the heart (Aird 2011; Khan et al. 2005). He explained that blood pumps with ventricular contraction through the lungs, then back to the heart, and then through the entire body. In the periphery it passes through “pores in the flesh” and returns to the heart through veins that increase in size as they approach the heart (Garber et al. 2008; Androutsos et al. 2012). Although he did not have the means to visualize it, he inferred that blood passed from the arteries to the veins through a small network of vessels, an hypothesis that was later proven by Marcelo Malpighi in 1661, who used microscopy to clearly prove a direct continuity between arteries and veins through capillaries (Motta 1998; Stapleton 2009). It took the scientific community 200 more years to establish that capillaries were lined by an unique epithelial cell type, which was termed *endothelium* by Wilhelm His in 1865 (Eliseyeva 2013).

For a long time, endothelial cells (ECs) were seen as a homogeneous population of cells only responsible for the formation of an inert barrier separating the vascular space from

the interstitium. However, studies performed over the last 40 years have clearly identified the endothelium as more than a barrier between blood and tissues. In 1977, Moncada and his colleagues published the first report indicating that the endothelium plays a central role in the control of vascular tone via the production of vasoactive substances (Moncada et al. 1977; Sandoo et al. 2010). Later, it was shown that it has a crucial role in regulating thrombosis, maintaining the adequate blood fluidity in different organs and parts of the vascular tree (Van Hinsbergh 2012; Yau et al. 2015) and in regulating coagulation by cross-talk with platelets (Marcus et al. 2003; Marcus et al. 1991; Galley 2004). This, together with the observations that activated endothelial cells play major roles in the pathophysiology of conditions such as inflammation and cancer (Ribatti 2008) has led to an exponential increase in studies focusing on endothelial cells and on their participation both in physiological and pathological conditions (Nachman 2012; Nachman & Jaffe 2004; Cines et al. 1998). In particular, the relation between endothelial cells and hematopoietic cells and their role as instructive players in the immune response to tumors has received increasing attention in recent years and will be the focus of this thesis.

1.2. BLOOD VESSEL FORMATION: VASCULOGENESIS AND ANGIOGENESIS

The cardiovascular system is the first functional organ system to develop in the vertebrate embryo in a process called vasculogenesis. The blood islands found in the extraembryonic yolk sac are the earliest vascular structures observed during development. These blood islands are believed to derive from the hemangioblasts which are commonly defined as precursors of endothelial and hematopoietic cells (Choi et al. 1998; Cao & Yao 2011). While the central cells within the blood islands give rise to embryonic hematopoietic cells, the peripheral cells differentiate into endothelial cells that will connect to form a primitive vascular plexus with lumen (**Figure 1.1**) (Risau & Flamme 1995; Weinstein 1999). Although vasculogenesis is a term usually employed to describe the formation of the primitive blood vessels inside the embryo and its surrounding membranes, it actually defines the *de novo* blood vessel formation. Several reports have shown that such phenomenon is not restricted to early embryogenesis, and instead occurs throughout adult

life, both in physiological and pathological conditions, in a process that is dependent on the recruitment of bone marrow-derived endothelial progenitor cells (EPCs) (Shi et al. 1998; Tepper et al. 2005; Asahara et al. 1999; Ribatti et al. 2001; Drake 2003).

After the primary vascular plexus is formed, it undergoes extensive remodeling where the specification of vessels to arteries and veins by distinct signals and underlying genetic programming takes place. The vascular wall of primitive blood vessels becomes structurally stabilized by mural cells that include vascular smooth muscle cells (SMC) for larger vessels and single pericytes around smaller vessels (Coultas et al. 2005; Adams & Alitalo 2007). Simultaneously, the primary plexus significantly expands in a process where new blood vessels arise from preexisting ones, called angiogenesis.

Angiogenesis can occur through intussusception or sprouting (**Figure 1.1**). Intussusceptive angiogenesis is the term used when a preexisting capillary is internally divided giving rise to daughter vessels (Djonov et al. 2000; Burri et al. 2004). It was first

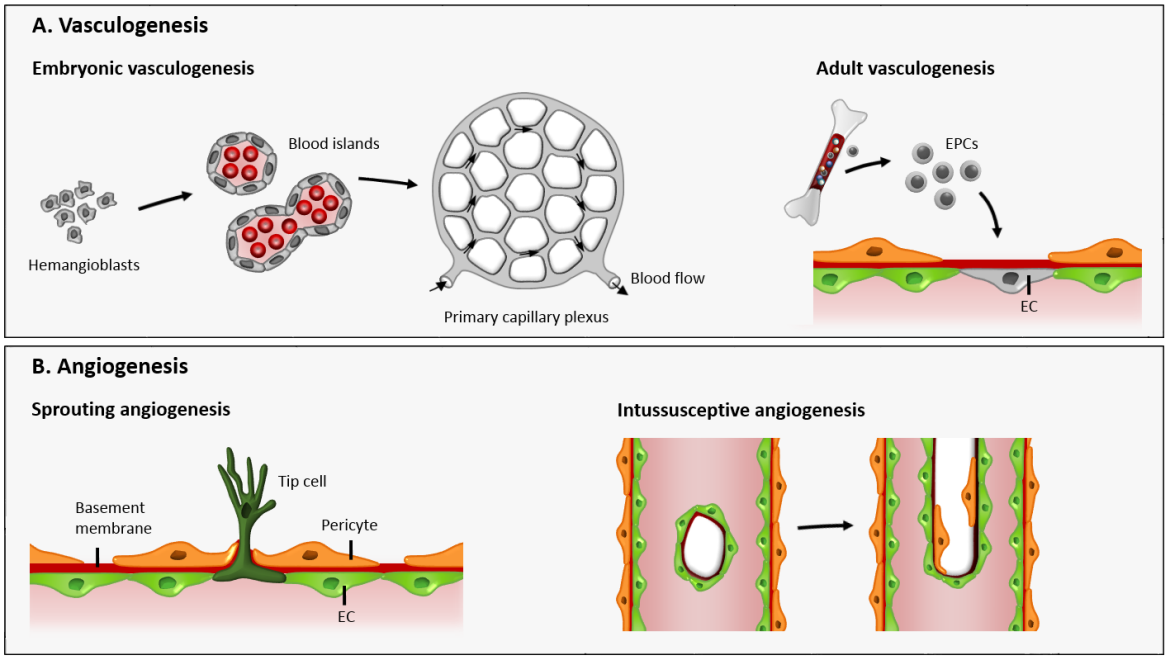

Figure 1.1. Mechanisms of blood vessel formation

(A) Mesodermal cells in the early embryo differentiate into endothelial and hematopoietic precursors (hemangioblasts) and aggregate to form blood islands. Fusion of blood islands leads to the vasculogenic formation of a honeycomb-shaped primary capillary plexi that presents arterial and venous specification of the endothelial cells. In the adult, endothelial progenitor cells (EPCs) are recruited from the bone marrow and differentiate into endothelial cells (ECs), contributing to vessel growth. **(B)** The expansion and remodeling of the vascular network occurs during angiogenesis by formation of new vessels from preexisting ones in a process called sprouting angiogenesis, or by internally splitting a vessel into two daughter vessels, which is called intussusceptive angiogenesis.

described in 1986 by Caduff et al. who showed that the postnatal transformation of the capillary network in the lungs was dependent on the insertion of new transcapillary pillars (Caduff et al. 1986). Such pillars are formed by the protrusion of opposing capillary walls into the lumen of a vessel with subsequent formation of an inter-endothelial zone of contact that, with the invasion of growth factors or cells, such as fibroblasts, leads to the formation of a channel in the vessel that eventually enlarges and splits the vessel into two (Burri et al. 2004). However, the predominant form of angiogenesis involves sprouting of new vessels from preexisting ones, by migration and proliferation of endothelial cells towards a pro-angiogenic stimulus.

1.2.1. Sprouting angiogenesis

Sprouting angiogenesis is a complex process, involving the expression of numerous genes by different cell types, all contributing to an integrated sequence of events (Conway et al. 2001; Geudens & Gerhardt 2011). It is induced by an inadequate supply of oxygen in tissues and organs. In response to hypoxia, the oxygen-sensible hypoxia-inducible factor 1 alpha (HIF1α) is no longer targeted for degradation by the von Hippel-Lindau (VHL) E3 ubiquitin ligase and accumulates inside the cells, leading to the subsequent expression and secretion of pro-angiogenic molecules coded by hypoxia-inducible genes. Examples include the vascular endothelial growth factor (VEGF), angiopoietin 2 (Angpt2), basic fibroblast growth factor (FGF2) and placental growth factor (PlGF) (Pugh & Ratcliffe 2003).

Sprouting endothelial cells

Sprouting angiogenesis is triggered by a pro-angiogenic stimulus, usually VEGF (described later in this Chapter), that induces the activation of quiescent endothelial cells (**Figure 1.2A and 1.2B**). Depending on each cell responsiveness to VEGF, different types of endothelial cells with unique morphologies are formed within a sprout (Gerhardt et al. 2003). The tip of the vascular sprout comprises a single endothelial cell, usually characterized by being the most responsive to VEGF due to its higher levels of VEGF receptor 2 (VEGFR2), that extends multiple long filopodia in a polarized manner (**Figure 1.2B**) (Wacker & Gerhardt 2011; Blanco & Gerhardt 2013). This cell, known as the tip cell, has specific proteolytic machinery, including the membrane-type 1 matrix metalloprotease

(MT1-MMP) (Yana et al. 2007), that favors the degradation of the surrounding basal lamina and presents a motile and invasive behavior that allows it to grow towards attractive cues (Ribatti & Crivellato 2012; Geudens & Gerhardt 2011). The endothelial cells that trail the tip cell and that are responsible for the elongation of the sprout are called stalk cells. Contrasting with the tip cells, stalk cells are highly proliferative and elongate the sprout as the tip cell migrates. In addition, stalk cells undergo morphological and positional rearrangements to form the lumen of the nascent sprout (**Figure 1.2C**) (Adams & Alitalo 2007; Iruela-Arispe & Davis 2009; Wacker & Gerhardt 2011).

Consistent with the distinct functions of each cell type, endothelial tip and stalk cells also differ in their expression profile. Although a tip cell-specific marker has not been identified, tip cells express high levels of Delta-like 4 (Dll4), VEGFR2, VEGFR3, Angpt2, platelet derived growth factor b (PDGFb) and unc-5 homolog b (Unc5b) and have low levels of Notch signaling activity. Contrastingly, stalk cells express Jagged1, VEGFR1 and Robo4 more strongly than tip cells (Gerhardt et al. 2003; Phng & Gerhardt 2009; Claxton & Fruttiger 2004; Lu et al. 2004; Tammela et al. 2008; Siekmann & Lawson 2007; Ribatti & Crivellato 2012).

Sprout fusion and lumen formation

To ensure that the newly formed sprouts are perfused, they need to have a functional lumen. As the sprouts elongate, lumen formation occurs either by fusion of growing vacuoles of one stalk cell with the vacuoles of adjacent stalk cells or by basal polarization and subsequent repulsion of the apical side of opposing stalk cells (Strilić et al. 2009; Lammert & Axnick 2012; Iruela-Arispe & Davis 2009).

Sprout formation and elongation is followed by a second crucial step towards the formation of a functional vascular network, the vessel anastomosis (**Figure 1.2C**). When a tip cell encounters the tips of other sprouts or existing capillaries, they suppress their motile and explorative behavior and fuse to create a new circuit that expands the vascular network (**Figure 1.2D**).

The fusion of migrating tip cells is mediated by Tie2- and Neurophilin-1-positive macrophages that act as chaperones by bridging neighboring tip cells (**Figure 1.2C**) (Fantin et al. 2010). VEGF-C expression by the tissue macrophages stimulates the VEGFR3- positive

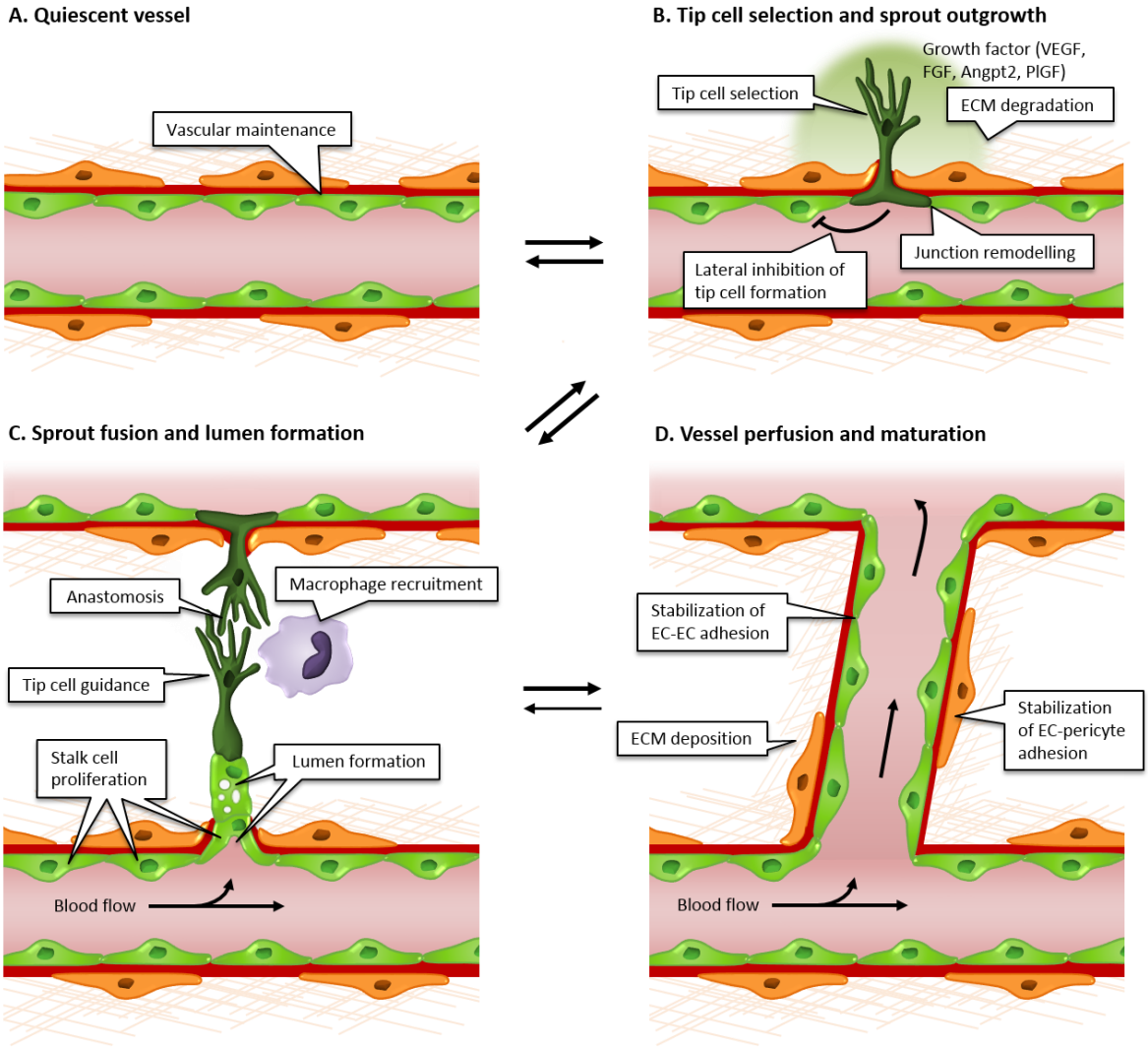

Figure 1.2. Cellular mechanisms of angiogenic sprouting

(A) In the absence of pro-angiogenic stimuli, endothelial cells (green) are retained in a quiescent state. **(B)** During angiogenesis, high levels of pro-angiogenic factors (such as VEGF, FGF, Angpt2 and PlGF) induce the selection of tip cells (dark green) for sprouting. Sprouting requires the induction of motile and invasive activity, modulation of cell-cell contacts and matrix metalloprotease-mediated degradation of extracellular matrix (ECM). The selected tip cells inhibit adjacent endothelial cells from responding to the pro-angiogenic signals and becoming tip cells. However, adjacent endothelial cells constantly compete for the tip cell position and they may shuffle and exchange positions with tip cells during angiogenic sprouting. **(C)** During sprout elongation, tip cells are followed by stalk cells (light green), which maintain connectivity with parental vessels and initiate vascular lumen formation through vacuole fusion. Upon contact with other sprouts, tip cell behavior is repressed and vessels fuse in a process called anastomosis, which is mediated by macrophages (purple) that act as chaperones by bridging the tip cells together. **(D)** Fusion processes establish a continuous and perfused lumen and subsequent maturation processes occur, such as the stabilization of endothelial cell-cell contacts, recruitment of pericytes and establishment of pericyte-endothelial cell contacts and matrix deposition that together re-establish a quiescent endothelial phenotype.

tip cells to turn on Notch target genes (VEGF and Notch signaling interaction will be addressed in Section 1.2.2), which decreases VEGF sensitivity in these cells and thus converts them into stalk cells, facilitating the assembly of functional microcirculatory loops (Tammela et al. 2011).

Once lumenised connections have been established and the previously poorly perfused tissues have a suitable oxygen delivery, paracrine VEGF expression is downregulated. This induces the endothelial cells to adopt a quiescent, immotile and non-proliferative phenotype, becoming phalanx cells (Mazzone et al. 2009). Although both the proliferative stalk cells and the quiescent phalanx cells are covered by supporting pericytes (Geudens & Gerhardt 2011), phalanx cells organize in a more regular, "cobblestone" appearance, show an increased expression of junctional molecules, such as ZO-1 and VE-Cadherin, and are surrounded by a more stable basement membrane, which improves tissue perfusion and oxygenation (De Bock et al. 2009; Mazzone et al. 2009).

1.2.2. Signaling pathways involved in sprouting angiogenesis

During the angiogenic process, the responses to the pro- and anti-angiogenic factors, and subsequent modulation of the phenotypic characteristics of the tip, stalk and phalanx cells, are tightly regulated. It depends on the interplay between several signaling pathways, including the VEGF and VEGFRs-, the Delta-Notch-, Angiopoietin/Tie receptor-, FGF- and PDGF-signaling pathways, that will be described in this section, with particular focus on the Delta-Notch signaling pathway.

VEGF signaling

The VEGF signaling pathway has been established as the key regulator of sprouting angiogenesis, both in physiological conditions and in disease. The VEGF family currently comprises seven members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and PlGF (Hoeben et al. 2004), each interacting differentially with specific tyrosine kinase receptors, VEGFR1, VEGFR2 and VEGFR3 and the non-signaling co-receptors neuropilin 1 (Nrp1) and Nrp2 (Roy et al. 2006; Hoeben et al. 2004). VEGF-A, often referred to simply as VEGF, is thought to be of singular importance in sprouting angiogenesis as, among other functions,

it promotes endothelial cell migration and proliferation and controls endothelial cell-cell junctions (Ferrara 2001; Phng & Gerhardt 2009). In fact, VEGF importance during vascularization is emphasized by the observations that both VEGF null and VEGF heterozygous mice are embryonically lethal (Carmeliet et al. 1996; Ferrara et al. 1996).

Most of the endothelial responses to VEGF are mediated through VEGFR2 (Waltenberger et al. 1994), which positively drives the mitogenic and chemotactic responses of endothelial cells to VEGF (Bernatchez et al. 2002). VRGFR-1 is a high-affinity receptor for VEGF, but its weak tyrosine-kinase activity turn it into a trap for VEGF by suppressing its availability to bind VEGFR2 (Shibuya 2001; Park et al. 1994; Kappas et al. 2008). However, genetic inactivation of either of the receptors causes embryonic lethality (Fong et al. 1995; Shalaby et al. 1995). Consistent with VEGFR1 function as a negative regulator of VEGF, *Vegfr1*$^{-/-}$ mice die due to severe vessel overgrowth and disorganization (Park et al. 1994), a phenotype that is rescued by the expression of the soluble form (that lacks the tyrosine kinase domain) of VEGFR-1 (Hiratsuka et al. 1998). On the other hand, VEGFR2 null mutation disturbs the vasculogenesis process, inhibiting the differentiation of endothelial cells and hematopoietic progenitor cells (Shalaby et al. 1995), suggesting it plays a key role in both vasculogenesis and angiogenesis.

The selection of the tip cell in the sprouting process depends mainly on the levels of VEGFR1 and VEGFR2 present on each cell (Jakobsson et al. 2010). Mosaic analysis have demonstrated that *Vegfr2*$^{+/-}$ cells have a disadvantage in adopting the tip cell position, whereas *Vegfr1*$^{+/-}$ cells have an advantage for the tip cell position. Moreover, as the levels of VEGF receptors are continuously altered in consequence of Notch activation (Williams et al. 2006), tip cell specification is highly transient, and the cells with higher VEGFR2 and lower VEGFR1 expression dynamically compete with and overtake their neighboring tip cells (Jakobsson et al. 2010).

Two other VEGF receptors are also involved in the tip cell selection and guidance process by interacting with VEGFR2: VEGFR3 and Nrp1. Similar to VEGFR1 and VEGFR2, VEGFR3 null mice also present an embryonic lethal phenotype and die at embryonic day (E) 10.5 due to defects in the primary vessel remodeling (Dumont et al. 1998). VEGFR3 is activated by the VEGF homologues VEGF-C and VEGF-D and is most strongly expressed in the leading tip cells during both mouse and zebrafish angiogenesis (Shawber et al. 2007;

Siekmann & Lawson 2007). Loss of VEGFR3 function in retinal endothelial cells results in hypersprouting (Zarkada et al. 2015; Tammela et al. 2011), probably through the loss of VEGFR2/VEGFR3 heterodimers, that negatively modulate VEGFR2 activity (Nilsson et al. 2010; Dixelius et al. 2003; Tammela et al. 2011; Zhang et al. 2010).

Nrp1 is expressed in the angiogenic vasculature, including tip cells, and enhances VEGF-mediated signaling by establishing interactions with VEGFR2. However, consistent with the observations that Nrp1 mutant mice exhibit defects in the heart, vasculature and nervous system and die at E10.5-12.5 (Kawasaki et al. 1999; Gu et al. 2003), this gene has been implicated in tip cell function and guidance in the embryo (Fantin et al. 2013; Jones et al. 2008; Gerhardt et al. 2004), in a process that is independent on the VEGFR2 co-receptor function (Aspalter et al. 2015).

Once tip cells are selected, they polarize and start moving towards the stimulus through the formation of filopodia and lamelopodia. Simultaneously, new sprouts are formed because of the proliferation and migration of adjacent stalk cells. Both the migration of tip cells and the proliferation of stalk cells is mediated by VEGF-induced VEGFR2 signaling. However, whereas tip cell migration depends on a gradient of VEGF, proliferation is regulated by its concentration, showing that VEGF distribution can regulate distinct cellular responses in defined populations of endothelial cells (Gerhardt et al. 2003; Geudens & Gerhardt 2011). Indeed, VEGFR2 activation in endothelial cells leads to activation of intracellular signaling cascades such as the mitogen-activated protein kinase (MAPK) and phosphatidyl-inositol-3 kinase (PI3K) (Karar & Maity 2011; Mavria et al. 2006). While the remodeling of the actin cytoskeleton required for the filopodia and lamelopodia formation seems to be induced by PI3K/Akt activation of Rho small GTPases, particularly CDC42 (Lamalice et al. 2004; Lamalice et al. 2007; Abraham et al. 2015), MAPK signaling has been implicated in endothelial cell proliferation (Meadows et al. 2001) and survival (Gupta et al. 1999; Berra et al. 2000). A balance in the activation of these two pathways might be what regulates the differential response to VEGF in tip and stalk cells.

Notch signaling

The Notch pathway is an evolutionarily conserved contact-dependent signaling machinery that is required for embryonic development, regulation of tissue homeostasis

and maintenance of the stem cell pool in adults. In fact, the Notch signaling pathway is a critical regulator of multiple cell fate decisions, tissue patterning and morphogenesis by coordinating proliferation, differentiation and survival in a broad range of cell types in a single organism and at different steps during cell lineage progression (J. Liu et al. 2010; Andersson et al. 2011).

In mammals, five canonical ligands, Delta-like 1 (Dll1), Delta-like 3 (Dll3), Delta-like 4 (Dll4), Jagged 1 (Jag1) and Jagged 2 (Jag2), interact with four Notch receptors (Notch1-Notch4). Both the Notch receptors and ligands are transmembrane proteins with large extracellular domains containing multiple epidermal growth factor (EGF)-like repeats. When receptor-ligand interactions are established between neighboring cells, two proteolytic cleavage events are triggered in the Notch receptor. The first cleavage is catalyzed by ADAM-family metalloproteases within the juxtamembrane region, whereas the second one is mediated by the γ-secretase complex within the single transmembrane domain of Notch receptors. The final cleavage releases the Notch intracellular domain (NICD) from the cell membrane which subsequently translocates to the nucleus where it interacts directly with the transcription factor RBPjk, also known as CSL (CBF1, Su(H), Lag2, after its mammalian, *Drosophila* and *Caenorhabditis elegans* orthologues).

In the absence of NICD, CSL functions as a transcriptional repressor by establishing interactions with a transcriptional corepressor complex. Following NICD binding to CSL, as a result of Notch activation, the corepressors are displaced, and the transcriptional coactivator Mastermind-like (Maml) is recruited. This NICD/CSL/Maml complex recruits additional coactivators to activate transcription of downstream target genes, such as hairy/enhancer of split (HES) and HES-related proteins (HEY/HRT/HERP), which in turn act as transcriptional regulators of further downstream genes (**Figure 1.3**) (Bray 2006; Blanco & Gerhardt 2013; Andersson et al. 2011; Kopan & Ilagan 2009; Holderfield & Hughes 2008; Phng & Gerhardt 2009). Several observations indicate that the Notch signaling pathway plays a key role at different stages of vascular development. The Notch ligands Dll1, Dll4 and Jag1 and the Notch receptors 1 and 4 are expressed in the vasculature (Hofmann & Iruela-Arispe 2007). Furthermore, deletion of genes involved in Notch signaling transduction, including receptors, ligands, transcription factors, downstream targets, and molecules that are involved in Notch processing lead to embryonic lethality in mice due to

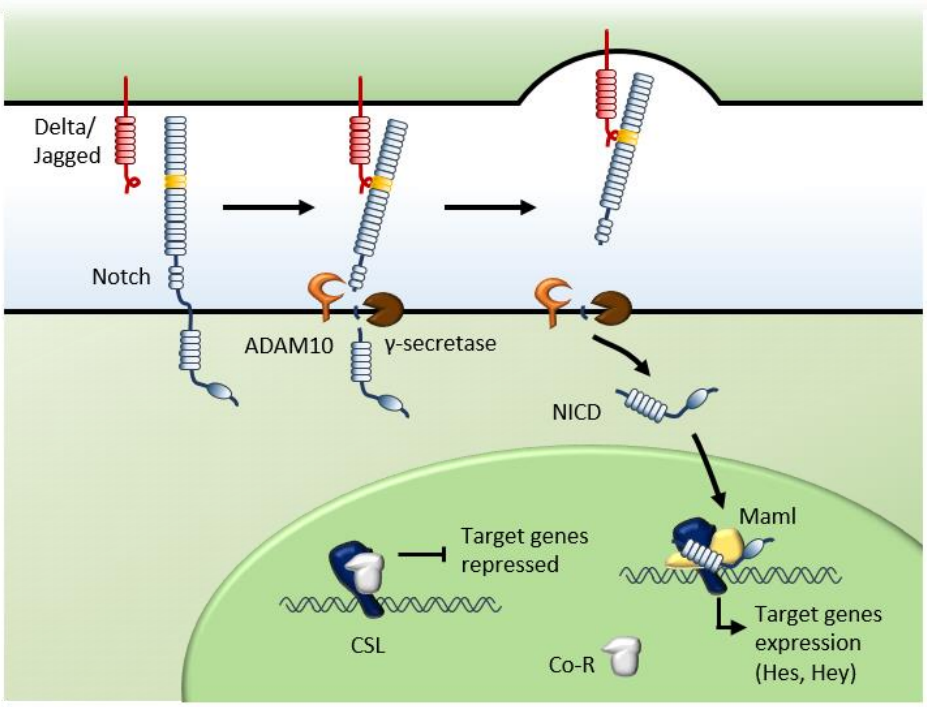

Figure 1.3. Canonical Notch signaling pathway

Delta/Jagged binding to the Notch receptor on an adjacent cell induces two proteolytic cleavages of the receptor catalyzed by ADAM10 and γ-secretase complex. This proteolytic processing mediates the release of Notch intracellular domain (NICD), which translocates to the nucleus and physically associates with DNA-binding CSL protein. In the absence of Notch activation, CSL recruits corepressors and silences transcription of Notch target genes. When bound to NICD, CSL recruits coactivators, such as Mastermind-like (Maml), and functions as transcriptional activator of Notch downstream target genes encoding various Hey and Hes family proteins.

severe vascular defects (**Table 1.I**). Knockout mice for Notch1, Notch1/Notch4 or the Notch ligand Jag1 die in utero between E9.5 and E10.5 of gestation, exhibiting major vascular abnormalities due to defects in the primary vascular plexus remodeling into a functional and organized vascular network (Krebs et al. 2000; Xue et al. 1999; Krebs et al. 2004; Gale et al. 2004; Duarte et al. 2004). Such defects seem to be caused particularly by the absence of those genes in endothelial cells because endothelial-specific knockout of Notch1 or Jag1, as well as expression of a constitutively active form of Notch4 (Notch4/int3) and Jag1 overexpression in an endothelial-specific manner, all cause embryonic lethality due to severe vascular defects similar to the ones found in Notch1-deficient mice (Limbourg 2005; Uyttendaele et al. 2001; Benedito et al. 2009; High et al. 2008). Moreover, although they are not found in the vasculature, Notch2 and Notch2/Notch3 knockout also induce vascular deficiencies that seem to relate with the lack of smooth muscle cell coverage, highlighting

the role of the Notch signaling pathway in several steps of vascular development (Hamada et al. 1999; Wang et al. 2012; McCright et al. 2001). Consistent with these findings, knockout mice for Adam10, components of the γ-secretase complex (*Ps1*$^{-/-}$/*Ps2*$^{-/-}$) or downstream targets of the Notch pathway (*Hey*$^{-/-}$/*Hey2*$^{-/-}$) all die during embryonic development with defects in the primary vascular plexus remodeling and in arteriovenous specification (Hartmann et al. 2002; Fischer et al. 2004; Kokubo et al. 2005; Herreman et al. 1999; Donoviel et al. 1999).

The Notch ligand Dll4 seems to be of particular importance in vascular development, which is highlighted by reports showing it is the only Notch pathway component causing haploinsufficiency in most genetic backgrounds (Duarte et al. 2004; Gale et al. 2004; Krebs et al. 2004), a penetrance only comparable to VEGF (Carmeliet et al. 1996). Knocking out one allele of Dll4 causes vascular remodeling defects and arteriovenous malformations such as the ones found in *Notch1*$^{-/-}$ mutants (Gale et al. 2004; Duarte et al. 2004; Krebs et al. 2004). Moreover, *Dll4*$^{+/-}$ mice have an increased number of vascular sprouts and vessel branches in the growing front of some vascular beds, such as the yolk sac (Gale et al. 2004; Suchting et al. 2007). The opposite phenotype is found in both ubiquitous and endothelial-specific Dll4 overexpression in mice, which cause decreased sprouting and increased arterialization (Trindade et al. 2008).

Table 1.I. Vascular defects associated with Notch-pathway mutants.

Mutated Gene	Phenotype
Receptors	
Notch1$^{-/-}$	Lethal at E10.5-11. Failure in remodeling the primary vascular plexus and disorganized embryonic vasculature. Impaired generation of HSCs from the hemangioblast and long-term definitive hematopoiesis (Krebs et al. 2000; Kumano et al. 2003; Hadland et al. 2004)
Notch2$^{-/-}$	Embryonic lethal at around E11.5. Reduced coverage of vascular smooth muscle cells (Hamada et al. 1999; Wang et al. 2012)
Notch2 hypomorphic allele (*Notch2*$^{del1/del1}$)	Embryonic lethality prior to E16.5. Defects in glomerular development in the kidney and in the development of the eye vasculature. Myocardial hypoplasia, hemorrhaging, and edema (McCright et al. 2001)
Jagged1$^{-/+}$/*Notch2*$^{del/+}$	Mimics human Alagille syndrome defects. Heart and kidney glomerular defects (McCright et al. 2002)
Notch3$^{-/-}$	Viable and fertile. Decreased retinal vascularization at early stages; Defective arterial specification and maturation of arterial vascular smooth muscle. (Krebs et al. 2003; Domenga et al. 2004; H. Liu et al. 2010)
Notch4$^{-/-}$	Normal development. Mice are viable and fertile (Krebs et al. 2000)

Mutated Gene	Phenotype
Notch1$^{-/-}$*/Notch4*$^{-/-}$	Lethal around E9.5. Failure in remodeling the primary vascular plexus and disorganized embryonic vasculature. Vascular defects were more severe than in Notch1$^{-/-}$ embryos (Krebs et al. 2000)
Notch2$^{-/-}$*/Notch3*$^{-/-}$	Lethal at E11.5. Enlarged vessels and thin vessel walls with reduced coverage of vascular smooth muscle cells (Wang et al. 2012)
EC-specific Notch1 knockout (*Tie2-Cre/Notch1*$^{lox/lox}$)	Embryonic lethal between E10.5-E11.5. Phenocopies Notch1-deficient mice, with absence of primary vascular plexus remodeling to form large and small blood vessels of the mature yolk sac (Limbourg 2005)
Constitutively active form of Notch4 (*int3*) regulated by the VEGFR-2 (*Flk1*) locus (*Flk1/Int3*)	Embryonic lethality between E9.5-10.5. Displayed disorganized vascular networks and dilated vessels. Failure in vascular remodeling and stabilization both in embryos and the yolk sac (Uyttendaele et al. 2001)
Notch4(int3)-inducible inactivation in ECs *(Tie2-tTA/TRE-Int3*)	Embryonic lethality. Expression in the adult causes blood vessel enlargement, defective arterialization and increased vascular smooth muscle cells. These phenotypes were reversed upon repression of *int3* expression (Carlson et al. 2005)
Ligands	
Jag1$^{-/-}$	Lethal between E9.5–11.5. Defective remodeling of the embryonic and yolk sac vasculature (Xue et al. 1999)
Dll1$^{lacZ/Dll1\ kineo}$ (combined Dll1 hypomorphic and null alleles)	Viable and fertile with loss of arterial identity (Sörensen et al. 2009)
Dll4$^{+/-}$	Embryonic lethality at E9.5-10.5. Phenocopies *Notch1*$^{-/-}$*/Notch4*$^{-/-}$ mutants. Absent remodeling of the yolk sac vasculature and defective arterial branching from the aorta. The number of vascular sprouts and vessel branches is increased in the growth front of some vascular beds, such as the yolk sac (Krebs et al. 2004; Gale et al. 2004; Duarte et al. 2004)
Inducible Dll4 overexpression (*chicken beta-actin (CAG)-Cre/TetO7-Dll4*)	Lethal at around E9.0-9.5. Failure in remodeling the primary vascular plexus; arterial cell identity is established in the venous compartment. Defects in vascular sprouting (Trindade et al. 2008)
EC-specific Dll4 overexpression (Tie2-rtTA-M2/TetO7-Dll4)	Lethal at around E10.5 presenting the same defects as ubiquitous Dll4 overexpression (Trindade et al. 2008)
EC-specific Jag1 knockout (*Tie1-Cre/Jag1*$^{lox/lox}$ or *Tie2-Cre/Jag1*$^{lox/lox}$)	Embryonic lethal at about E10.5. Defects in smooth muscle cell development in both embryonic and yolk sac blood vessels, accompanied with loss of arterial specification (Benedito et al. 2009; High et al. 2008)
EC-specific Jag1 overexpression (*VE-Cadherin-tTA/TetO-Jag1*)	Lethal before E16.5. Extensive hemorrhaging in the skin. (Benedito et al. 2009)
Regulators	
Rbpsuh$^{-/-}$	Embryonic lethal before E9.5. Vascular defects similar to *Notch1*$^{-/-}$*/Notch4*$^{-/-}$ double mutants. Loss of arterial specification (Oka et al. 1995; Krebs et al. 2004)
Adam10$^{-/-}$	Lethal at around E9.5. Vascular defects resemble the ones found in *Notch1*$^{-/-}$*/Notch4*$^{-/-}$ mice (Hartmann et al. 2002)
EC-specific Adam10 knockout (*Tie2-Cre/Adam10*$^{lox/lox}$)	Embryonic lethal at E11.5. Exhibit large-caliber vessels on the liver surface and myocardium. Augmented expansion of erythroid precursors in the BM, but increased hemolysis (Glomski et al. 2011)
Ps1$^{-/-}$ (component of the γ-secretase complex)	Lethal at late gestation or after birth. Vascular remodeling failure in stomach and skin; reduction of cerebral sprouting in the brain with increased diameter of sprouting capillaries and brain hemorrhages (Nakajima et al. 2003)
Ps2$^{-/-}$ (component of the γ-secretase complex)	Viable and fertile. Hemorrhages and pulmonary fibrosis found in adult mice (Herreman et al. 1999)
Ps1$^{-/-}$*/Ps2*$^{-/-}$	Embryonic lethality after E9.5. Failure in remodeling the yolk sac vasculature and disorganized embryonic vasculature (Herreman et al. 1999; Donoviel et al. 1999)

Mutated Gene	Phenotype
Target genes	
Hey1 $^{-/-}$	Viable and fertile. No obvious phenotypic anomaly (Fischer et al. 2004)
Hey2 $^{-/-}$	Mice die within 10 days after birth. Ventricular septal defects (Sakata et al. 2002; Gessler et al. 2002; Donovan et al. 2002)
Hey1$^{-/-}$/*Hey2*$^{-/-}$	Lethal between E9.5-11.5. Failure in remodeling the primitive vascular plexus in the yolk sac, massive hemorrhages and absence of large embryonic blood vessels (Fischer et al. 2004; Kokubo et al. 2005)

In the last decade, studies in the mouse retina, in zebrafish intersegmental vessels and in 3D endothelial cell culture sprouting assays have demonstrated that the Dll4/Notch signaling is the major regulator of the tip and stalk cell specification process (Hellström et al. 2007; Lobov et al. 2011; Siekmann & Lawson 2007; Suchting et al. 2007; Leslie et al. 2007; Patel 2005). The mechanism by which Notch imposes differential behavior in endothelial cells that are exposed to similar doses of a pro-angiogenic stimulus is directly connected to VEGF signaling, and a negative regulatory loop is established between these two pathways. VEGF signaling in endothelial cells regulates Dll4 expression both *in vivo* and *in vitro*. Studies in retina where VEGF is blocked, either with VEGF-Trap or with a soluble form of VEGFR1, both decreased Dll4 mRNA expression (Suchting et al. 2007; Lobov et al. 2007). Conversely, VEGF stimulation in human umbilical cord vein endothelial cells (HUVECs) increases Dll4 expression (Ridgway et al. 2006), a phenotype that was also observed in human tumor samples where VEGF and Dll4 expression were found to be directly correlated (Patel 2005).

In addition to VEGF acting upstream of Dll4, it has become clear that Dll4-induced Notch signaling activation negatively regulates VEGF signaling by regulating the expression of the different VEGF receptors (VEGFR1, VEGFR2, VEGFR3 and Nrp1). Heterozygous mice for Dll4 (*Dll4*$^{+/-}$) were shown to have a downregulation in VEGFR2 accompanied with VEGFR1 upregulation in retinal vessels, which correlated with increased sprouting (Suchting et al. 2007; Jakobsson et al. 2010). *In vitro* studies have also helped to shed some light on the Notch-dependent VEGF signaling regulation. Notch activation in HUVECs decreases both VEGFR2 and Nrp1 mRNA expression (Williams et al. 2006; Ridgway et al. 2006) and simultaneously upregulates of VEGFR1 and VEGFR3 (Harrington et al. 2008; Funahashi et al. 2010; Shawber et al. 2007), which altogether renders the cells less responsive to VEGF.

Considering the experimental observations, the current models propose that VEGF and Notch signaling specify the balance between tip and stalk cells through a tight cooperation. Small stochastic differences in VEGFR2 expression or activity provide a competitive advantage to some endothelial cells to acquire the tip phenotype. Consistently, VEGFR2 activation directly translates into Dll4 expression and, in turn, endothelial cells that express more Dll4 activate Notch signaling in the neighbor cells, effectively inhibiting them from becoming tip cells through modulation of the VEGF receptors levels (**Figure 1.4**). Disrupting either of these pathways has profound effects in sprouting angiogenesis. Specifically, retinas from *Dll4*$^{+/-}$ mice as well as wild-type retinas treated with a pharmacological Notch signaling inhibitor or with a Dll4 neutralizing antibody (Dll4-Fc), all present a hypersprouting phenotype (Suchting et al. 2007; Lobov et al. 2007). Similarly, endothelial deletion of *Vegfr3* also resulted in marked excessive branching and filopodia projection (Tammela et al. 2011), whereas endothelial-specific *Vegfr2* deletion severely impaired sprouting (Zarkada et al. 2015).

Jag1 is another Notch ligand that is also involved in tip cell regulation (Benedito et al. 2009). Unlike what was reported for Dll4, endothelial Notch activity is higher in the absence of Jag1, indicating that Jag1 negatively regulates Notch activity. Such regulation is dependent on the glycosylation of the Notch receptor by the glycosyltransferase Fringe. Whereas the unglycosylated receptor is activated by both Jag1 and Dll4, Notch glycosylation decreases its activation in response to Jagged ligands (Yang 2004). Consistently, Jag1 is most prominently expressed in stalk cells (Hofmann & Iruela-Arispe 2007), and functions as a competitive inhibitor of the Dll4-induced Notch activation in tip cells, rendering them a further advantage on stalk cells (**Figure 1.4**) (Benedito et al. 2009). This differential function of Dll4 and Jag1 illustrate how the Notch pathway regulates sprouting through distinct mechanisms, and not only by the amount of individual ligands in each cell type.

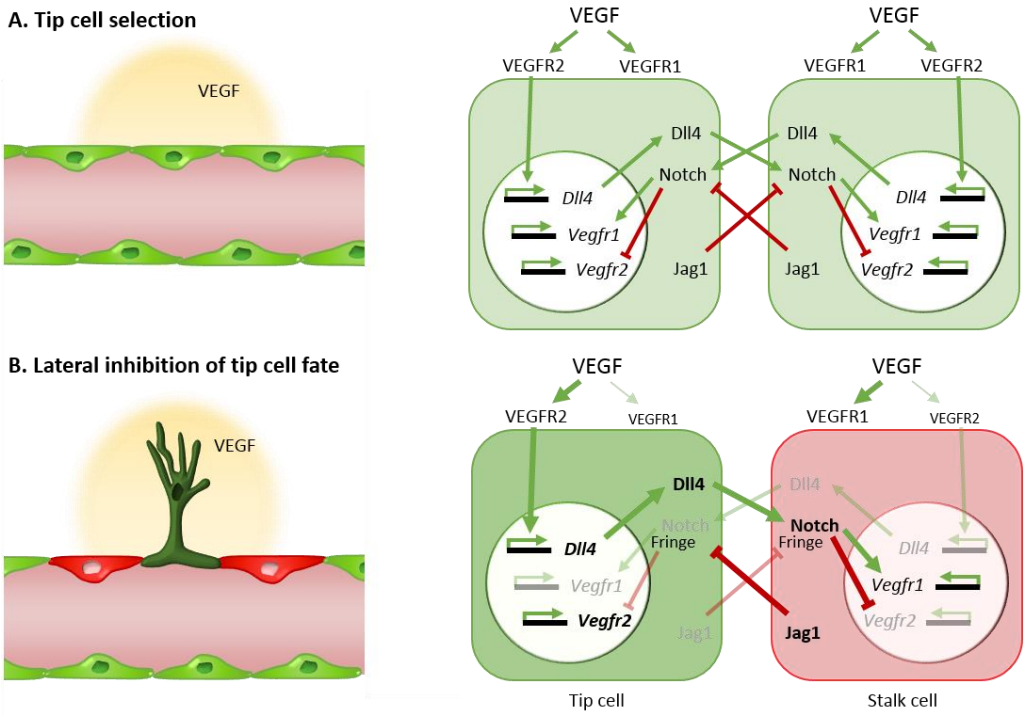

Figure 1.4. Notch signaling regulates endothelial tip/stalk cell specification

(A) During sprouting angiogenesis, quiescent endothelial cells (light green) become activated by VEGF stimulation and compete for tip cell specification via bilateral Dll4-Notch signaling involving a VEGF-Notch feedback loop. **(B)** Under VEGF stimulation, Dll4 expression is upregulated in the tip cells (dark green) which, in turn, activates Notch signaling in the adjacent cells (red), suppressing the tip cell phenotype. Notch signaling activation reduces VEGFR2 levels, while stimulating the expression of the decoy receptor VEGFR1, inducing the acquisition of an endothelial stalk cell phenotype. In contrast, low Notch activity in the tip cell allows high expression of VEGFR2, but low VEGFR1, inducing continuous expression of Dll4 and the maintenance of the tip cell phenotype. Contrary to Dll4, Jagged1 is expressed by the stalk cells and antagonizes Dll4-Notch signaling in the sprouting front when the Notch receptor is modified by the glycosyltransferase Fringe. Glycosylation of Notch receptors by Fringe enhances Dll4-Notch signaling, but suppresses Jag1-Notch signaling, which ensures that the Notch receptors in tip cells do not get activated by Jag1 on stalk cells, strengthening the differential Notch activation in tip and stalk cells.

Angiopoietin-Tie signaling

The angiopoietin family consists of two tyrosine kinase receptors, Tie1 and Tie2, and three secreted ligands, Angiopoietin 1 (Angpt1) to Angpt4. Tie2 is predominantly expressed on vascular endothelial cells and binds to all the Angiopoietins, whereas Tie1, with no known ligand, binds to Tie2 and regulates its activity (Seegar et al. 2010; Saharinen et al. 2005). Angpt1 and Angpt2 both have important roles in angiogenesis. However, the nature of their contributions is very distinct (**Figure 1.5**). Angpt1 is constitutively expressed in a variety of adult tissues and is expressed by pericytes, smooth muscle cells (SMCs) and

fibroblasts. On the other hand, Angpt2 expression in the adult is restricted to sites of vascular remodeling and is primarily produced by endothelial cells (Maisonpierre et al. 1997).

Under physiological conditions, Angpt1-dependent Tie2 activation promotes the stabilization and integrity of existing vessels not only via recruitment of mural cells, but also through regulation of vascular permeability. The distribution of VE-Cadherin in adherens junctions is regulated by VEGF and Angpt1 in opposing manners. VEGF-induced VEGFR2 activation triggers VE-Cadherin internalization leading to disrupted intercellular junctions and increased vessel permeability (Gavard 2009). Angpt1 however, inhibits the internalization of VE-Cadherin, maintaining tight endothelial cell contacts and thus vessel integrity (Thurston et al. 2000; Gavard et al. 2008).

Endothelial Angpt2 antagonizes Angpt1 activity, and thereby promotes the dissociation of pericytes from pre-existing vessels and increases vascular permeability. This facilitates the infiltration of cytokines and proteases that further expose and sensitize endothelial cells to pro-angiogenic signals, such as VEGF (Huang et al. 2010). Angpt2 binding to specific integrins in activated endothelial cells further enhances VEGF-induced sprouting in a Tie2-independent manner (Felcht et al. 2012). Furthermore, VEGF itself, as well as insulin-like growth factor (IGF1) and PDGFb, can also induce Angpt2 expression creating a positive regulatory loop (Oh et al. 1999; Hu & Cheng 2009). During sprouting angiogenesis, Angpt2 is most strongly, but not exclusively, expressed in the tip of the sprouting vessels (Toro et al. 2011; Felcht et al. 2012). Through its antagonizing role of Angpt1, Angpt2 might help maintain the unstable nature of the newly formed vascular sprouts by preventing pericyte recruitment to that area (Huang et al. 2010).

Although *Angpt2* null mutation does not cause embryonic lethality (Gale et al. 2002), mice deficient for Angpt1 or Tie2 or overexpressing Angpt2 all die in utero between E9.5 and E10.5 with severe vascular defects, namely the loss mural cells lining the endothelium (Dumont et al. 1994; Davis et al. 1996; Sato et al. 1995; Maisonpierre et al. 1997), which emphasizes the crucial role of Angpt1-induced signaling in vessel stabilization.

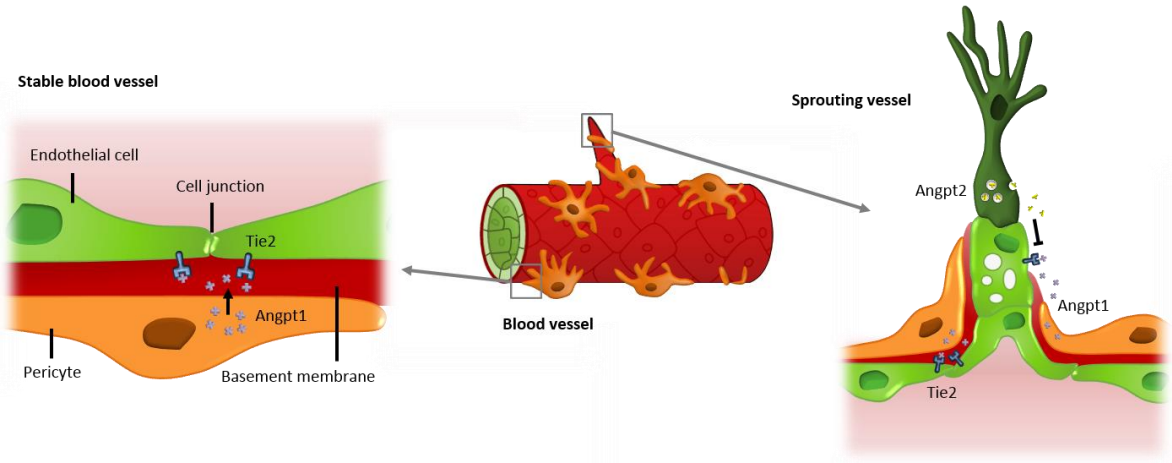

Figure 1.5. Angpt-Tie system regulates mural cell recruitment and vascular permeability

In stable vessels, endothelial cells express Tie2 receptor, which becomes activated by pericyte-derived Angpt1. The quiescent endothelial phenotype is maintained by constitutive Angpt1-Tie2 signaling, which clusters Tie2 at the endothelial cell-cell junctions, promoting junction stabilization, mural cell recruitment, basement membrane deposition and survival. In vessels undergoing angiogenesis, Angpt2, which is predominantly expressed in the tip cells of the sprouting vessels, is released from the Weibel-Palade bodies where it is endogenously stored, and antagonizes Angpt1-Tie2 signaling, promoting the dissociation of pericytes and increasing vascular permeability. In sprouting angiogenesis, Tie2 expression in the stalk cells and its Angpt1-dependent signaling is required for attenuation of angiogenesis and stabilization of the newly formed sprouts.

FGF signaling

The FGF family consists of 22 structurally-related proteins that act on different cell types, including endothelial cells, by signaling through one or several of the cell-surface tyrosine kinase FGF receptors (FGFR; FGFR1-4). The two most studied FGFs are the acidic and basic FGFs (aFGF and bFGF), also known as FGF1 and FGF2 (Itoh & Ornitz 2004).

FGFs stimulate new vessel formation and vessel maturation by driving endothelial cell proliferation and migration, promoting extracellular matrix degradation, altering intercellular adhesion and vascular integrity by modulating VE-Cadherin at adherens junctions, and affecting integrin expression (Presta et al. 2005; Hatanaka et al. 2012; Murakami et al. 2008).

During angiogenesis, an intimate cross-talk exists between FGF and VEGF signaling pathways. Several experimental evidences suggest that FGF2 might require the activation of the VEGF signaling pathway to promote angiogenesis (Seghezzi et al. 1998; Tille et al. 2001; Fujii & Kuwano 2010), and conversely, VEGF may require FGF2 for its angiogenic potential (Presta et al. 2005). In fact, FGF2 induces Angpt2 in endothelial cells (Fujii &

Kuwano 2010), which is required for mural cell detachment in vascular sprouting and branching, suggesting that VEGF and FGF signaling pathways have synergistic effects on angiogenesis (Presta et al. 2005; Stratman et al. 2011).

PDGFb signaling

During angiogenesis, PDGFb expression is largely restricted to endothelial cells, whereas PDGFRβ expression occurs in SMCs and pericytes (Lindahl et al. 1997; Hellström et al. 1999). PDGFb is released from angiogenic endothelial cells and binds to PDGFRβ expressed on the surface of pericytes, inducing their recruitment to the newly formed sprouts (Lindblom et al. 2003). In fact, knocking out *Pdgfb* or *Pdgfrb* in mice leads to perinatal lethality caused by microvascular leakage and hemorrhage (Levéen et al. 1994; Soriano 1994) as a result of total loss of mural cell coverage (Lindahl et al. 1997; Hellström et al. 1999).

In the angiogenic vasculature, PDGBb expression is higher in tip cells than in stalk cells (Gerhardt et al. 2003; Lindblom et al. 2003). The observation that pericytes are present only slightly behind the tip cells, leads to the postulation that the release of PDGFb by these cells immediately attracts pericytes to the emerging angiogenic sprouts, thereby stabilizing them (Gerhardt & Betsholtz 2003). PDGFb also seems to be particularly more expressed in developing arteries than in their venous counterparts, concomitant with the higher need for pericyte and SMC recruitment in those vessels (Hellström et al. 1999; Lindahl et al. 1997; Gerhardt & Betsholtz 2003).

1.2.3. Vessel maturation

Formation of a functional vessel with circulatory blood flow requires maturation processes, such as the differentiation of vessels in arteries or veins and vessel stabilization by recruitment of mural cells. The arteriovenous differentiation process starts early in the embryo during the remodeling of the primary vascular plexus (Torres-Vázquez et al. 2003). In addition to its role in regulating sprouting angiogenesis, VEGF is also implicated in arteriovenous specification through VEGFR2/Nrp1-induced signaling. This induces the activation of Notch receptors through Dll4 and the expression of Notch target genes, such

as the transcription factors HES, HEY and the most commonly used arterial marker Ephrin B2 (Iso et al. 2006; Adams & Alitalo 2007). Expression of the transcription factors HES and HEY strengthens the arterial cell fate via transcriptional repression of venous-specific genes, such as the venous marker EphB4 (Iso et al. 2006). The role of Notch signaling in arterial specification is emphasized by the embryonic lethality found in *Dll4*$^{+/-}$, *Rbpjk*$^{-/-}$ or *Hey1*$^{-/-}$*/Hey2*$^{-/-}$ mice, where major defects in arterial differentiation were found (**Table 1.I**).

Determination of a venous endothelial cell fate is essentially regulated by chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) (You et al. 2005). COUP-TFII, a member of the orphan nuclear factor superfamily, is expressed in the venous endothelium and inhibits the Notch signaling cascade, preventing the expression of Ephrin B2 and the HEY/HES transcription factors, thereby promoting the expression of the Ephrin B2 receptor, EphB4 (You et al. 2005; Adams & Alitalo 2007). Consistently, knockout mice for COUP-TFII have veins with arterial characteristics, expressing Notch-pathway components, Nrp1 and Ephrin B2, whereas mis-expression of COUP-TFII in arterial endothelial cells suppresses the expression of those arterial markers, modifying the arteries into vein-like vessels (You et al. 2005).

Hemodynamic forces such as blood pressure and the direction of blood flow also seem to influence the identity of a vessel. Particularly, high pressure flow induces the vessels to acquire a more arterial identity (Kwei et al. 2004), whereas experimental manipulation of the flow pattern in the yolk sac of chick embryos alters the arteriovenous network and regulates the expression of arterial markers, such as Nrp1 and Ephrin B2 (le Noble 2003).

The process that describes the transition from an actively growing vascular bed into a quiescent and functional network is termed maturation. It is through this process that stalk cells acquire a phalanx phenotype, involving several modifications in the endothelial cells, such as the suppression of endothelial proliferation, maturation of tight junctions and recruitment of mural cells – pericytes and SMCs (Herbert & Stainier 2011). Pericytes establish direct intercellular contact with endothelial cells and cover the walls of capillaries and immature blood vessels, whereas SMCs cover mature and larger diameter vessels, such as arteries and veins (Bergers & Song 2005).

Initially, PDGFb is released from angiogenic endothelial cells and attracts PDGFRβ-positive pericytes that are incorporated in the vessel wall of the newly formed and immature vessels. These pericytes establish direct cell-cell contacts with the endothelial cells and they both form the basement membrane of the microvasculature (Mandarino et al. 1993). Furthermore, Angpt1 release from mural cells activates the Tie2 receptor in endothelial cells, promoting further recruitment of mural cells (Suri et al. 1996), as well as endothelial cell quiescence and survival (Brindle 2006). Although the mechanism is still poorly understood, subsequent differentiation of pericytes into SMCs can be induced in part via the release of transforming growth factor β1 (TGFβ1) by endothelial cells. TGFβ-induced activation of the TGFβ receptors (TGFβR) expressed in mural cells induces SMC differentiation (Chen & Lechleider 2004) and leads to the formation of SMC-covered quiescent mature vessels.

In contrast with pericytes, that communicate with endothelial cells through direct physical contact and paracrine signaling pathways, SMCs are not integrated into the basement membrane and do not signal directly to endothelial cells (Gerhardt & Betsholtz 2003). Multiple sheets of SMCs with intercalated layers of extracellular matrix and elastic fibers are formed in larger arteries and veins. Arteries and arterioles, however, present a more extensive coverage of SMCs than the respective veins and venules, consistent with the higher requirements for artery contractility and capacity of withstanding higher blood pressures (Adams & Alitalo 2007; Gerhardt & Betsholtz 2003).

An unambiguous identification of pericytes and SMCs that allows the understanding of the relationships between both types of mural cells is still largely hampered by the absence of specific molecular markers. PDGFRβ, desmin, the proteoglycan NG2, α-smooth-muscle actin (SMA) and the promoter trap transgene XlacZ4, among others, have been described to label pericytes. However, some of these markers are also expressed by SMCs and other cell types, and none of them recognizes all the pericytes populations (Gerhardt & Betsholtz 2003; Armulik 2005).

1.3. ENDOTHELIAL CELL GENERAL PROPERTIES

Once a functional vessel network is established, quiescent endothelial cells are characterized by specific properties that help maintain vessel homeostasis. The establishment and maintenance of tight interactions between adjacent endothelial cells and the deposition of the basement membrane are of critical importance to determine both endothelial cell polarity and vessel permeability.

1.3.1. Endothelial cell junctions

Endothelial cells adhere to adjacent endothelial cells through a complex network of adhesion proteins. Such proteins are linked to specific intracellular partners that not only mediate anchorage to the actin cytoskeleton, therefore stabilizing junctions, but also function as signaling structures that communicate cell position, protect cells from apoptosis, limit cell growth by contact inhibition and regulate endothelial homeostasis (Dejana 2004). Junctional complexes can trigger intracellular signaling directly, by interacting with signaling proteins or specific receptors, or indirectly limiting the nuclear translocation of transcription factors, by tethering and retaining them at the cell membrane (González-Mariscal et al. 2008; Braga 2002; Wheelock & Johnson 2003).

Changes in endothelial cell-cell junctions lead to modifications in the normal architecture of the vessel wall and compromise endothelial interactions with circulating blood cells, which might contribute to dysfunctions in disease states, such as altered permeability, ischemia, inflammation and carcinogenesis.

Molecular organization of endothelial cell junctions

Junctions in endothelial and epithelial cells share common features. Two major types of junctions have been described in both cell types: adherens junctions and tight junctions (**Figure 1.6**). However, the organization of the different types of junctions is more variable in endothelial cells and their topology is less restricted than in epithelial cells.

Adherens and tight junctions are both formed of transmembrane proteins that promote homophilic cell-cell interactions and form a pericellular zipper-like structure along the cell border. However, they are formed by different molecular components and have

distinct functions. Whereas adherens junctions have a crucial role in maintaining vessel integrity, tight junctions play a role in regulating the barrier functions of endothelial cells, such as leukocyte diapedesis and the passage of solutes and ions through the vessel wall (Bazzoni et al. 2004; Wallez & Huber 2008). Several other proteins, such as platelet endothelial cell adhesion molecule (PECAM1/CD31), intercellular adhesion molecule 1 (ICAM1), Muc18 (CD146) and Endoglin (CD105), are also concentrated in the intercellular clefts and are implicated in cell-cell interactions and in maintaining tissue integrity, but are not specifically confined to adherens and tight junctions (Bazzoni et al. 2004).

Endothelial junctions are highly variable among different segments of the vascular network, considering the permeability requirements of each organ and each branch of the vascular tree. Adherens junctions are ubiquitous in all types of vessels. Tight junctions, however, vary in their complexity. For instance, in the brain, where strict control of permeability between the blood and the nervous system is required, junctions are well developed and rich in tight junction complexes. Furthermore, tight junctions are well developed in arterial endothelial cells, which are subjected to high flow rates, but less complex in capillaries and even less in venules, which are the primary sites of leukocyte extravasation (Wallez & Huber 2008; Dejana 2004).

Adherens junctions

At adherens junctions, adhesion is mediated by transmembrane proteins of the cadherin family. Endothelial cells express comparable high levels of two cadherins: a cell-type-specific cadherin, vascular-endothelial cadherin (VE-Cadherin) and neuronal cadherin (N-Cadherin), which is also present in other cell types such as smooth muscle cells and neural cells. Nonetheless, in most cases, N-Cadherin is excluded from cell-cell contacts and only VE-Cadherin is clustered at these structures (**Figure 1.6**). Other members of the cadherin family, such as P-Cadherin and T-Cadherin are also expressed in different types of endothelial cells (Bazzoni et al. 2004).

VE-Cadherin depletion in mice results in embryonic death at midgestation (E11.5), with severe defects in sprouting angiogenesis (Gory-Fauré et al. 1999). In fact, VE-Cadherin is required for the establishment of correct endothelial cell polarity and lumen formation in large vessels of the mouse embryo (Strilić et al. 2009) and prevents the disassembly of

nascent blood vessels (Crosby et al. 2005). Furthermore, it is implicated in the inhibition of sprouting activity when tip cells connect with each other. Tip cells lacking VE-Cadherin do not sense the cell-cell contact and instead keep searching for other connections (Lenard et al. 2013).

VE-Cadherin binds catenins, in particular p120, β-catenin, and plakoglobin. In turn, both β-catenin and plakoglobin bind to α-catenin, which regulates the actin cytoskeleton, and therefore modulates cell shape and motility (Wallez & Huber 2008). Furthermore, a stable interaction of the VE-Cadherin-catenins complex with the actin cytoskeleton is also involved in the stabilization of endothelial cell junctions and in the reduction of their dynamic opening (Giannotta et al. 2013).

Permeability-increasing agents, such as VEGF or tumor necrosis factor (TNF-α), or leukocyte adhesion to endothelial cells via ICAM1, can destabilize the junctions by inducing the tyrosine phosphorylation of VE-Cadherin and its binding partners, leading to VE-Cadherin internalization and cytoskeleton remodeling. VEGF signaling and VE-Cadherin are tightly linked (**Figure 1.6**). In quiescent endothelial cells, VE-Cadherin binds VEGFR2 and retains it at the cell membrane, where it is quickly dephosphorylated. This inhibits the VEGF-induced proliferation signal through the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) and protects cells from apoptosis by activating the phosphatidylinositol 3-Kinase (PI3K)/Akt signaling pathway (Giannotta et al. 2013; Dejana et al. 2008). An endothelial-specific receptor protein phosphatase (VE-PTP), responsible for dephosphorylating VE-Cadherin and keeping it at the cell membrane, has also been reported to dephosphorylate VEGFR2 in stalk cells. Thus, VE-PTP dissociation from VE-Cadherin, which is triggered by VEGF or the binding of leukocytes, induces VEGFR2 activity and increases VE-Cadherin tyrosine phosphorylation and internalization, leading to increased vascular permeability (Hayashi et al. 2013; Bazzoni et al. 2004; Broermann et al. 2011).

Tight junctions

Tight junctions are composed by three types of structural transmembrane components: the IgG-like family junctional adhesion molecules (JAMs) and members of the claudin and occludin families (**Figure 1.6**).

Occludin is exclusively localized at the tight junctions of epithelial and endothelial cells. Expression of occludin in the endothelium is correlated with the permeability of different branches of the vascular tree. For example, it is expressed at much higher levels in brain endothelial cells than in endothelial cells from other tissues that do not require such a strict control of permeability (Hirase et al. 1997). However, occludin does not seem to be essential for tight junction formation, as *occludin*-deficient mice still have tight junctional strands and barrier function (Saitou et al. 2000).

The claudin family is comprised of at least 24 members that establish homophilic and heterophilic interactions through their extracellular domains, forming the tight junction strands. Each claudin exhibit a specific organ and tissue distribution. Endothelial cells highly express Claudin 5 but its role is apparently redundant, as mice deficient for Claudin 5 have a size selectivity impairment in the blood brain barrier function, but still exert barrier function for molecules larger than 800 Da (Nitta 2003).

Junction adhesion molecule-A (JAM-A) and its related family members JAM-B, JAM-C, endothelial cell-selective adhesion molecule (ESAM), cocksackie and adenovirus receptor and JAM4 are transmembrane glycoproteins that can be engaged in homophilic and heterophilic adhesion. All JAM family members and ESAM are expressed in endothelial cells. However, JAMs do not induce the formation of tight junctional strands when expressed in fibroblasts, and inactivation of JAM genes in mice does not affect the normal development of the vascular system in the embryo, suggesting that they do not have a crucial role in tight junction formation (Dejana et al. 2009; Weber et al. 2007). Nonetheless, JAMs seem to play a role in the establishment of apical-basal polarity, since JAM-A binds to the partitioning-defective protein 3 (Par3) and tether the Par3/aPKC/Par6 polarity complex to the tight junctions. JAMs are not exclusively found in cells that form tight junctions, and the expression of these molecules in leukocytes, for instance, modulates their transmigration through endothelial cells (Ebnet et al. 2004).

Occludin, claudins and JAMs do not establish direct interactions. Thus, to incorporate and include these molecules in tight junctional strands, cytoplasmic binding partners are required. An important group of tight junctional scaffolding proteins are the zona occludens proteins ZO-1, ZO-2 and ZO-3 that interact together and anchor claudins, occludin and JAMs to the cytoskeleton by binding directly to actin (**Figure 1.6**) (Niessen

2007). ZO proteins also establish numerous protein-protein interactions that cluster diverse kinases, phosphatases, small G proteins and nuclear transcription factors at the tight junctions. Interactions of the scaffolding proteins or of the transmembrane members of the tight junctions themselves with other signaling proteins modulate not only the tight junction paracellular transport, but also cell survival, proliferation and apoptosis, through differential activation of signaling pathways, such as the MAPK and the PI3K/Akt pathways (González-Mariscal et al. 2008).

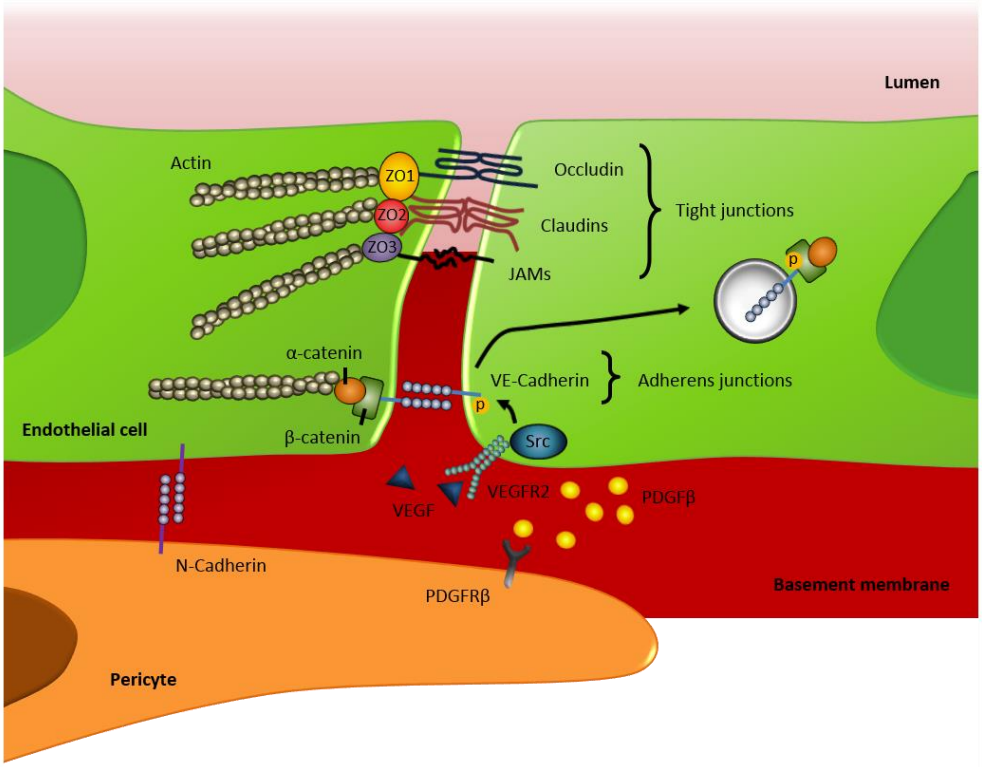

Figure 1.6. Adhesive proteins involved in the establishment of endothelial cell-cell and endothelial-pericyte interactions

Occludin, claudins and JAMs from adjacent endothelial cells establish homophilic interactions and are the main tansmembrane components of the tight junctions. Since no direct interactions were found between these adhesive proteins, cytoplasmic binding partners, such as ZO1, ZO2 and ZO3, function as scaffolding molecules, and provide a direct link to the actin cytoskeleton, resulting in the formation of tight junctional strands. The endothelial barrier is also maintained by the integrity of the adherens junctions through homophilic interactions between VE-Cadherin molecules expressed in adjacent endothelial cells. VE-Cadherin is connected to the actin cytoskeleton by establishing interactions with catenins that are able to bind actin. VEGFR2 associates with VE-Cadherin, and when activated by VEGF, this receptor dimerizes and activates the Src kinase, resulting in VE-Cadherin phosphorylation and consequent internalization, disrupting the adherens junctions and increasing vascular permeability. The secretion of PDGFβ by endothelial cells recruits PDGFRβ-expressing pericytes that establish direct interactions with endothelial cells through homophilic N-Cadherin binding.

1.3.2. Endothelial-pericyte interactions

In addition to the paracrine signaling pathways that exist between endothelial cells and pericytes (described in Section 1.2.3), pericytes also communicate with endothelial cells by direct physical contact through interruptions in the basement membrane in which both endothelial cells and pericytes are embedded, enabling adhesion, recognition and signaling. Pericytes are located in the basal side of endothelial cells and in general a single pericyte covers several endothelial cells incompletely (**Figure 1.5**) (Diaz-Flores et al. 1991). The number and size of pericyte-endothelial contacts varies between tissues, but up to 1000 contacts have been described for a single endothelial cell. Furthermore, as a pericyte contacts with several endothelial cells, it integrates signals along the length of the vessel (Bergers & Song 2005). The endothelial-pericyte contacts show different structural classes of junctions, such as peg and socket, adherens junctions and gap junctions. In the peg-socket type of contacts, pericyte cytoplasmic fingers (pegs) are inserted into endothelial invaginations (pockets), and may touch the endothelium, establishing gap and adherens junctions between both cell types (Armulik 2005).

Gap junctions are formed by transmembrane channels composed by proteins of the connexin family. These channels, found in the pericyte and endothelial cell membranes are lined up with one another through the intercellular space, providing direct connections between the cytoplasms of pericytes and endothelial cells, and allowing the passage of ions, metabolites and small molecules, including signaling molecules (Díaz-Flores et al. 2009). In fact, gap junctions appear to be involved in endothelium-induced mural cell differentiation through TGBβ signaling activation, as demonstrated in *Connexin 43*-knockout mice (Hirschi 2003).

Similar to the adherens junctions established between two endothelial cells, endothelial-pericyte adherens junctions connect the cytoskeleton of pericytes and endothelial cells by means of cadherins and catenins, and are important for contact inhibition and to provide strong mechanical attachment, supporting the transmission of strong mechanical contractile forces. Particularly, N-Cadherin is the key junction protein for the establishment of endothelial-pericyte interactions (**Figure 1.6**), and consistently, several signaling pathways known to be important for vascular maturation, such as TGFβ, Notch and sphingosine-1-phosphate (S1P), regulate N-Cadherin in heterotypic cell

interactions between pericytes and endothelial cells (Armulik et al. 2011; Winkler et al. 2011).

1.3.3. Extracellular matrix

The extracellular matrix (ECM), a scaffold of proteins secreted and organized by endothelial and mural cells, plays a substantial role in the maintenance of vessel integrity. In addition to the mechanical support provided by the ECM to endothelial cells, through adhesive interactions with integrins on the endothelial cell surface, ECM molecules directly influence endothelial cell morphogenesis by modulating cytoskeletal organization and by mediating adhesion, migration and proliferation. Moreover, ECM components also regulate pro- and anti-angiogenic factors, by shaping their gradients, regulating endothelial functions in an indirect manner (Red-Horse et al. 2007). Thus, the ECM affects several fundamental aspects of endothelial cell biology through both structural support and modulation of signaling pathways. The diversity of ECM components in the endothelial cell microenvironment and of mechanisms that control the synthesis and degradation of ECM suggests that structurally and functionally distinct ECMs are formed, exerting precise control over the neovascularization or blood vessel maturation demands of a specific site (Davis & Senger 2005).

The basement membrane, in which both endothelial cells and pericytes are embedded, is a specialized form of ECM consisting of a tight meshwork mainly comprised of laminin, collagen, fibronectin and proteoglycans. Laminins are the primary determinants of the basement membrane assembly whereas the other basement membrane components are accessory components (Murakami 2012). However, homozygous mice for collagen IV display dilated blood vessels and unstable basement matrix that becomes disrupted when placed under mechanical stresses, leading to embryonic lethality (Pöschl et al. 2004).

Laminins are essential for keeping vessel stability. They interact with and activate laminin-binding integrins expressed in endothelial cells, suppressing proliferation and regulating activation, by inhibiting the MAPK and NFκB pathways, which promotes endothelial cell stabilization. Furthermore, Laminin1-induced activation of the GTPase Rac and protein kinase A (PKA) and suppression of Rho activity also maintain endothelial cells

in a quiescent state (Davis & Senger 2005). Similarly, collagen seems to work as a negative regulator of angiogenesis, since proteolytic fragments of collagen XVIII and collagen IV inhibit endothelial cell proliferation and function as endogenous inhibitors of angiogenesis (O'Reilly et al. 1997; Maeshima et al. 2002).

The ability of endothelial and mural cells to directly remodel their adjacent ECM components is therefore of critical importance for the formation, stabilization and remodeling of vascular networks.

1.4. DISRUPTION OF ENDOTHELIAL CELL PROPERTIES

The establishment of endothelial cell-cell and endothelial-pericyte interactions and the formation of an adequate extracellular matrix (basement membrane) are critical steps of vessel formation and play a crucial role in vessel maintenance and integrity. Nonetheless, vessels from different vascular sites present variations in these properties that seem to correlate with their different functions. For instance, as stated previously, the brain and the retina are lined by continuous endothelial cells connected by complex tight junctions that help maintain the blood-brain barrier; the liver, spleen and bone marrow have sinusoidal blood vessels lined by discontinuous endothelial cells that allow cellular trafficking through the intercellular gaps; the kidneys, endocrine glands and intestinal villi, on the other hand, are lined by fenestrated endothelial cells that promote the selective permeability required for efficient filtering, secretion and absorption (Dejana 1996; Garlanda & Dejana 1997). In addition, endothelial cells participate in other physiological processes, such as the control of vasomotor tone, balance of pro- and anti-inflammatory mediators and immune surveillance. This control is also dependent on the type of vessel in which the endothelial cells are inserted and on their anatomical localization. For example, the arteriolar endothelium is primarily responsible for the modulation of the vasomotor tone; the post-capillary venous endothelium regulates leukocyte trafficking; and endothelial cells balance local hemostasis in different vascular beds through the expression of site-specific anti-coagulants and pro-coagulants (Aird 2006). It is therefore clear that endothelial cells adapt the morphological and molecular properties that better suit the demands of a specific organ or site. Alterations in endothelial cells with the disruption of

these specific properties play a central role in the pathogenesis of a wide range of diseases. The endothelium considerably contributes to the onset and progression of diseases such as stroke, heart disease, diabetes, inflammation, tumor growth and metastasis (Rajendran et al. 2013). In this Section, the contribution of endothelial cells in the onset of inflammation and cancer will be addressed.

1.4.1. Endothelial cells in inflammation

The process of inflammation involves the rapid recruitment and activation of neutrophils, and results from the activation of endothelial cells, which is defined as the acquisition of new capacities by quiescent endothelial cells. In a physiological state, endothelial cells do not interact with leukocytes, as leukocyte-interactive proteins, such as P-Selectin and chemokines, are internalized into vesicles and the transcription of adhesion molecules, such as E-Selectin, vascular cell adhesion molecule 1 (VCAM1) and ICAM1, is suppressed. Although nitric oxide (NO) can suppress the synthesis of these molecules, inhibiting endothelial-leukocyte interactions indirectly, it also has direct effects on leukocytes, preventing their activation to motile cells capable of transendothelial migration (Pober & Sessa 2007). NO also contributes to the quiescence of endothelial cells and the maintenance of blood vessels by preventing platelet activation and aggregation and providing a stimulus for smooth muscle cell relaxation, leading to vasodilation and maintenance of tissue blood flow (Pate et al. 2010).

Endothelial cell activation

The activation of endothelial cells that ultimately leads to leukocyte recruitment can be triggered by a wide variety of mechanisms. These include radiation, pathogen recognition pathways (such as toll-like receptors and lipopolysaccharide [LPS]), reactive oxygen intermediates, by-products of the coagulation pathway and molecules generated during inflammation, such as complement and cytokines (Pate et al. 2010). Activated endothelial cells control the development of inflammation at several levels. First, an increase of local blood flow is observed. This is triggered by an enhanced production of vasodilators, such as prostacyclin, by arteriolar endothelial cells, and is essential for leukocyte delivery to the site of inflammation. In type I endothelial cell activation,

prostacyclin production begins with the ligand-specific activation of heterodimeric G-protein-coupled receptor (GPCR), such as thrombin and histamine H1 receptors, leading to transient elevations in cytosolic free Ca^{2+}. This induces cellular phospholipase A2 (cPLA2)-derived cleavage of membrane phosphatidylcholine and release of arachidonic acid, which is rapidly converted into prostacyclin in a reaction that is initiated by cyclooxygenase 1 (Cox1). Additionally, cytosolic Ca^{2+} ions also activate nitric oxide synthase 3 (NOS3) to produce NO, which synergizes with prostacyclin (Pober & Sessa 2007). Contrasting to type I, type II endothelial activation requires new gene expression. Pro-inflammatory cytokines derived mainly from activated leukocytes, such as TNF and interleukin 1 (IL1), induce the expression of a high-throughput Cox1 isoform (named Cox2), thereby greatly augmenting prostacyclin production (Pober et al. 2009). Therefore, the two types of endothelial activation responses cooperate, not only to increase the efficiency and duration of the local vasodilation, but also in other aspects of inflammation (**Figure 1.7**).

The second step of inflammation requires a localized leakage of plasma-proteins, such as fibrinogen and fibronectin, into the tissue, accounting for the swelling of inflamed tissues. Vascular leakiness of plasma proteins is also caused by the increase in cytosolic Ca^{2+} free ions, which interact with the Rho pathways and lead to the contraction of actin filaments (Alexander 2000). As these filaments are attached to the endothelial tight and adherens junctions, their contraction results in the opening of gaps between adjacent cells (Pober & Sessa 2007). These responses are stronger in post-capillary venules, where tight junctions are less complex and GPCRs are maximally expressed (Bazzoni et al. 2004; Heltianu et al. 1982). In type II activation, TNF and IL1 also induce the leakage of plasma proteins through the rearrangement of the actin and tubulin cytoskeleton, in an NF-κB- and protein synthesis-dependent manner (Sprague & Khalil 2010). Plasma proteins leak from the blood into the tissues where they form a provisional matrix that supports invading leukocyte survival, attachment and migration (Pober et al. 2009).

The following step requires localized recruitment and activation of circulating leukocytes with consequent infiltration into the infected or damaged tissues. The rise in intracellular Ca^{2+} in endothelial cells is also critical for leukocyte recruitment as it induces the exocytosis of the vesicles containing P-Selectin and its localization into the luminal cell surface, inducing the tethering of circulating neutrophils (Panés & Granger 1998).

Additionally, it increases the acetylation of lysophosphatidylcholine, a by-product of phosphatidylcholine cleavage, generating an endothelial cell-derived form of platelet-activating factor (PAF) (Whatley et al. 1990; Tolins et al. 1991) that induces leukocyte activation and rapid mobilization of integrins, such as the CD11/CD18 complex, into the leukocyte surface (Panés & Granger 1998). Following type II activation, the pro-inflammatory cytokines induce expression of other adhesion molecules, such as E-Selectin, and chemokines, such as CXC-chemokine ligand 8 (CXCL8; also known as Il8) (**Figure 1.7**). Similar to PAF, CXCL8 also increase the expression of CD11b/CD18 on leukocytes, inducing

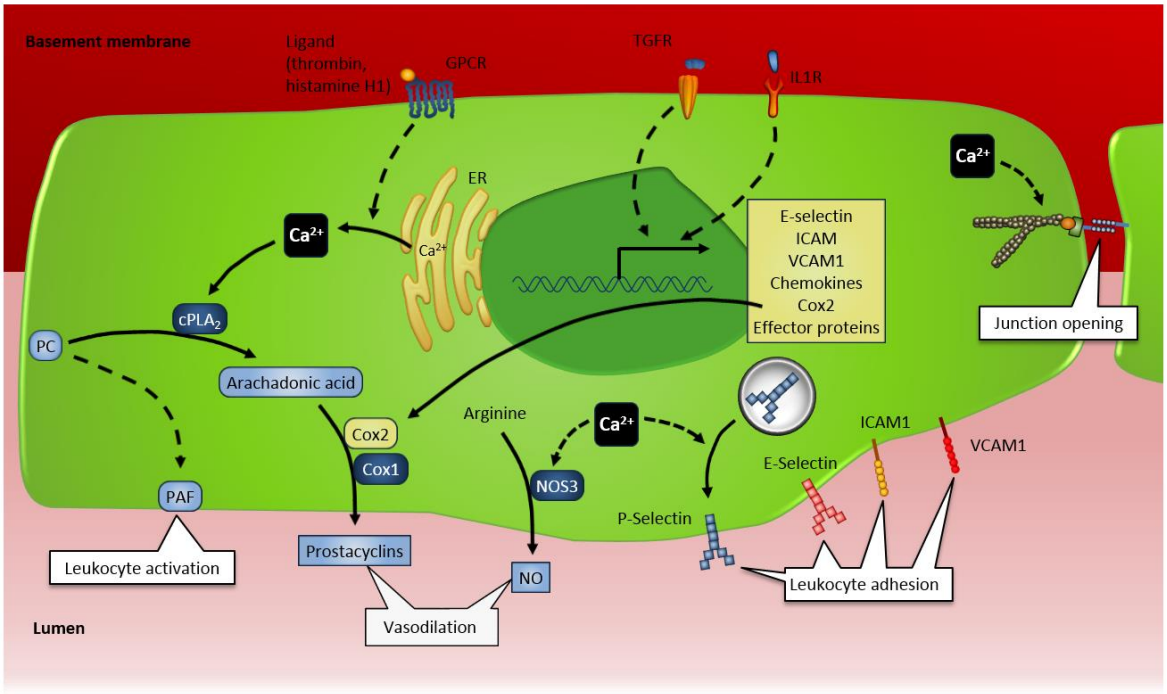

Figure 1.7. Endothelial cell activation

In type I activation of endothelial cells (blue boxes), the binding of a ligand to a heterodimeric G-protein-coupled receptor (GPCR), triggers the release of Ca^{2+} ions from the endoplasmic reticulum (ER). The increase in cytosolic Ca^{2+} leads to the formation of platelet-activating factor (PAF) and prostacyclins, which deliver activating signals to leukocytes, initiating endothelial transmigration, and induce relaxation of smooth muscle cells, respectively. Elevated levels of Ca^{2+} also induce actin contraction and junction opening, inducing vascular leakage of plasma proteins; promote the exocytosis of the vesicles containing P-Selectin; and activate nitric-oxide synthase 3 (NOS3), which generates nitric oxide (NO). NO synergize with prostacyclin to relax vascular smooth muscle, increasing blood flow and leukocyte delivery to the tissues. In type II activation (yellow boxes), the binding of inflammatory cytokines to the respective receptors lead to the transcription of specific genes that have a pro-inflammatory effect. Among these are adhesion molecules that bind leukocytes, such as E-Selectin, intracellular adhesion molecule 1 (ICAM1) and vascular cell-adhesion molecule 1 (VCAM1); chemokines and enzymes, such as Cox2, and other effector proteins that reorganize actin filaments and open endothelial junctions, promoting vascular leakage.

their attachment to endothelial cells and migration into the tissue (Panés & Granger 1998; Pober & Sessa 2007).

Although type I activation induces a rapid response because it does not require new gene expression, GPCRs become desensitized 10 to 20 minutes following their activation, limiting the degree of inflammation and neutrophil extravasation. Type II activation provides a somewhat slower response, but generates a more sustained and stronger inflammatory response (Pober & Sessa 2007). Moreover, the responses to TNF and/or IL1 are able to evolve over time. Within 6 to 24 hours after cytokine-mediated activation, E-Selectin expression decreases, and other adhesion molecules, such as VCAM1 and ICAM1, and chemokines, such as monocyte chemoattractant protein 1 (MCP1), become highly expressed (Munro et al. 1989; Panés & Granger 1998). These changes induce the replacement of neutrophils as the predominant infiltrating leukocyte to leukocytes of the monocyte/macrophage lineage (Henderson et al. 2003).

Endothelial cell dysfunction

By 24 hours, inflammation might be exacerbated by leukocyte-mediated endothelial cell damage, particularly in the capillaries. TNF and IL1, when combined with other mediators, such as interferon-γ (IFNγ), also contribute to endothelial cell injury by inducing endothelial cell death. TNF is also involved in the inhibition of NOS3 and the anticoagulant thrombomodulin synthesis (Ramseyer et al. 2012; Okamoto et al. 2012). Additionally, TNF and IL1 induce the synthesis of tissue factor (TF), which results in activation of the coagulation cascade (Bevilacqua et al. 1986). Together, such modifications in endothelial cells hamper endothelial cell-derived control of vascular tone, vessel permeability, coagulation and leukocyte quiescence. Failure of endothelial cells to adequately perform any of these basal functions is known as ‘endothelial cell dysfunction’ (Pober & Sessa 2007).

Removal of the inflammatory stimulus, such as the elimination of an infection, induces the decrease in cytokine production causing the resolution of type II activation. However, if an acute inflammatory response fails to eradicate the tissue infection or damage, the inflammatory process will evolve to a more chronic form, in which an adaptive immune response is activated, and specialized effector cells are recruited. Endothelial cells modify

the synthesis of adhesion molecules to recruit effector and memory T cells and induce their activation and transendothelial migration by functioning as antigen presenting cells (APCs).

Immune-driven angiogenesis

The generation of new blood vessels is essential for the sustained survival of inflammatory cells within the tissue, both in acute and chronic inflammation, and is a common aspect in most immune-mediated conditions. As inflammation evolves, the leukocytes accumulated in the infected or damaged tissue, particularly the activated T cells and mononuclear phagocytes, produce endothelial growth factors such as VEGF, FGF, Angpt1 and Angpt2, inducing angiogenesis (Pober & Sessa 2007). Thus, the contribution of endothelial cells to inflammation has two distinct phases. First, functional changes that include vessel dilation, increase permeability, and leukocyte diapedesis, prevail. Then, structural changes such as vessel remodeling and expansion are observed.

Three signaling pathways are usually activated in endothelial cells during the process of angiogenesis: the ERK1/ERK2 pathway, the PI3K/Akt pathway and the Ca^{2+}/Phospholipase Cγ (PLCγ) pathway (Shibuya & Claesson-Welsh 2006). Ligand binding to FGFR1 or VEGFR2 activates all three signaling pathways. However, Angpt1 binding to Tie2 selectively activates the PI3K/Akt or the ERK pathway. Although Angpt1-induced Tie2 activation is responsible for stabilization of endothelial cell-cell contacts and for survival signals of resting endothelial cells through the activation of the PI3K/Akt pathway, it can also contribute to the proliferation and migration of angiogenic endothelial cells by activating the ERK signaling pathway (Huang et al. 2010). TNF can also act as a pro-angiogenic cytokine by binding to TNFR2 and leading to VEGFR2 phosphorylation with consequent activation of the PI3K/Akt pathway (R. Zhang et al. 2003). TNF and IL1 can also contribute to angiogenesis through the production of cytokines, such as CXCL8, that present angiogenic properties (Heidemann 2003). Furthermore, by inducing Cox2 expression, they lead to the activation of FGFR1 through Cox2-derived prostaglandins, which also contribute to angiogenesis (Wang & DuBois 2004).

In chronic inflammatory disorders, the angiogenic and inflammation processes become co-dependent: the angiogenic vessels have increased expression of integrins that recruit and activate leukocytes; the leukocytes express angiogenic growth factors and

cytokines that contribute for endothelial cell activation and growth, and the increased blood supply to the inflamed tissues sustains the viability of the inflammatory cells (Danese et al. 2007).

Endothelial cells in immune dysfunction and disease

A number of widespread and devastating chronic diseases, including atherosclerosis, rheumatoid arthritis, type 2 diabetes, and Alzheimer's disease, have a pathophysiological important inflammatory component (Tabas & Glass 2013). Consistent with the significant number of functions exerted in inflammation, endothelial cells play a crucial role in the development of these immune-related diseases. Inhibition of factors that promote angiogenesis may reduce tissue inflammation and prevent disease progression (Yoo & Kwon 2013).

In rheumatoid arthritis, synovial endothelial cells are activated and show increased leakiness, apoptosis and angiogenesis. These changes result in leukocyte recruitment, edema, and pannus formation. A pannus is a membrane of granulation tissue composed of fibroblast-like mesenchymal cells, macrophage-like cells and other inflammatory cells that release collagenolytic enzymes (Konttinen et al. 1998). Pannus invasion of the articular cartilage and bone, together with the stimulation of TNF, IL1, PlGF, and prostaglandins by macrophages, ultimately causes cartilage destruction and bone erosion (McCoy et al. 2002; Kay & Calabrese 2009; Yoo et al. 2009). Endothelial dysfunction is also recognized as an important element of cardiovascular diseases (CVD) in rheumatoid arthritis, which account for 30-50% of all deaths (Totoson et al. 2014).

Similar events occur in atherosclerosis. Endothelial dysfunction is also thought to represent an initial step in the pathogenesis of this disease, inducing adhesion of blood leukocytes and directed migration of the bound leukocytes into the tunica intima, the inner layer of the arterial vessels, in response to chemoattractants, such as MCP1 (Libby et al. 2011; Steyers & Miller 2014). Following migration into the intima, monocytes differentiate into macrophages that uptake and accumulate lipids, resulting in their transformation into foam cells. This initiates the atherosclerotic lesion and begins a cascade of events with an enhanced release of inflammatory cytokines, ultimately inducing the formation of atherosclerotic plaques (Mudau et al. 2012).

Chronic inflammation and abnormalities in the vascular system of the brain also contribute to the onset and progression of neurodegenerative events in Alzheimer's disease. Brain endothelial cells in Alzheimer's patients express high levels of inflammatory adhesion molecules, such as MCP1 and ICAM1, suggesting endothelial dysfunction. Additionally, a higher amount of inflammatory cytokines, such as TNFα, TGFβ and IL1, is also found in the brain microvasculature of those patients (Grammas 2011).

Inhibition of adhesion molecules or chemokines from the dysfunctional endothelium, as well as inhibition of endothelial growth factors or molecules from the angiogenic signaling pathways, may reduce inflammatory responses and prevent disease progression. TNF, for example, is both a pro-angiogenic factor and a type II activator of endothelial cells. TNF-specific antibodies or a soluble TNF receptor have been used for disorders in which angiogenesis contributes for inflammation, such as rheumatoid arthritis (Bilsborough et al. 2006). Consistently, blocking VEGF-induced signaling pathways, using a soluble form of VEGFR1, significantly reduced joint destruction and the severity of the disease in a rheumatoid arthritis mouse model (Paleolog 2002). TNF inhibition, however, exacerbates the damage in diseases like congestive heart failure that require angiogenesis for the recovery and repair processes (Gullestad & Aukrust 2005). Similar to TNF, Cox2 also has a pro-angiogenic effect and is an effector of the type II activation of endothelial cells. Cox2 inhibitors have been shown to decrease the extent of both rheumatoid arthritis and atherosclerosis (Belton & Fitzgerald 2003; Woods et al. 2003). Treatment with non-steroidal anti-inflammatory drugs (NSAIDs), non-selective Cox1 and Cox2 inhibitors, also protects patients from the development of Alzheimer's disease (Ajmone-Cat et al. 2010). Preventing the interactions of leukocytes with endothelial cells, using antibodies against endothelial and leukocyte adhesion molecules, such as inhibition of VLA4 or the CD11/CD18 complex in leukocytes, or ICAM1 or VCAM1 blockade in endothelial cells, has been shown to be a promising approach for controlling inflammation and autoimmune diseases (Yusuf-Makagiansar et al. 2002).

A variety of drugs have been developed to inhibit inflammation, targeting either the leukocytes themselves, endothelial cells or even cells from the tissue microenvironment. Many aspects of the endothelial signaling pathways and inflammation mediators are common to leukocytes and, as a consequence, several anti-inflammatory drugs target both

cell types. However, due to the variety of effects exerted by the inflammatory mediators, such as TNF and VEGF, this isn't always an advantage and may lead to harmful effects when inhibitors of these mediators are used (Pober & Sessa 2007).

1.4.2. Endothelial cells in cancer

Similar to normal tissues, the growth of new capillary vessels is required in tumors to support their nutrient and oxygen needs and to discard waste products, and is now accepted as one of the 'hallmarks of cancer'(Hanahan & Weinberg 2011). The hypothesis that tumor growth is angiogenesis dependent and that blocking angiogenesis might be a means of controlling tumor growth was first postulated by Judah Folkman in 1971 (Folkman 1971). Although an oncogenic event may allow tumor cells to evade surveillance or enhance their survival, tumors are unable to grow more than 1 mm^3 without a supporting vascular network, remaining small and dormant. This observations suggested that tumor cells and vascular endothelial cells are part of a highly integrated ecosystem and that an "angiogenic switch" occurs, where endothelial cells can be induced by signals derived from tumor cells to progress from a quiescent state to a rapid growth phase (Folkman et al. 1989). A considerable body of evidence indicates that the angiogenic switch is controlled by a balance of pro- and anti-angiogenic factors that either induce or oppose angiogenesis (Bergers & Benjamin 2003), and that tumor cells can tilt this balance towards a pro-angiogenic state, to stimulate vessel growth (Weis & Cheresh 2011).

Folkman's discoveries also inspired many researchers to look for antiangiogenic molecules and to design anti-angiogenic strategies for cancer treatment (Ribatti 2008). However, as the pro- and anti-angiogenic molecules, such as VEGF and thrombospondin (TSP1), have unique functions on multiple cell types, a better understanding of how these molecules are regulated is essential for the design and development of effective anti-angiogenic therapies.

The tumor vasculature

Tumor blood vessels are morphologically different from their normal counterparts (**Figure 1.8**). Imaging of a variety of tumors has revealed chaotic networks of tortuous and dilated vessels lacking the normal hierarchical organization of arteries, arterioles,

capillaries, venules and veins (Nagy et al. 2009). Due to the overproduction of pro-angiogenic growth factors, such as VEGF, by tumor cells and other cells from the tumor microenvironment, the tumor vasculature lacks the tight endothelial monolayer essential for normal barrier function, resulting in vascular leakiness and hemorrhage (Bergers & Benjamin 2003). Poor vessel stability may also be caused by less abundant and more loosely attached pericytes (**Figure 1.8B**) (Baluk et al. 2005). These changes in vessel stability may affect blood flow. Some vessels are not perfused with blood at all. Others have an irregular blood flow that may alternate directions leading to dysfunctional capillaries (Nagy et al. 2009). This architectural dysregulation contributes to intratumoral hypoxia and acidosis, and increased interstitial pressure, which can promote the outward growth of tumors. Hypoxia can then directly contribute to tumor aggressiveness not only by inducing angiogenesis but also by promoting metabolic switches and multidrug resistance in cancer cells (Peng & Liu 2015). Other tumor endothelial phenotypic abnormalities include augmented integrin expression (Weis & Cheresh 2011), dysregulated leukocyte adhesion (Castermans & Griffioen 2007), and abnormal mechanosensing (Ghosh et al. 2008), anomalies that have been described in dysfunctional endothelial cells in vascular disease (Steyers & Miller 2014; Danese et al. 2007)

Tumors can be quite heterogeneous in their vascular patterns. Some tumors rely heavily on vasculogenesis, recruiting endothelial progenitor cells from the bone marrow. Tumor-derived VEGF can signal on VEGFR1 and VEGFR2 that are expressed on hematopoietic progenitor cells and endothelial progenitor cells, respectively, and mobilize them into circulation, from where they are recruited into the vascular network of some tumor types (Rafii et al. 2008). The first evidence that blood vessel growth occurred through both angiogenesis and vasculogenesis was provided by David Lyden and his colleagues, who showed that impairing the recruitment of bone-marrow derived hematopoietic and endothelial progenitor cells blocked tumor angiogenesis and growth (Lyden et al. 2001). Several lines of evidence also support the idea that tumor-derived cells can integrate into the vessel wall and form vascular channels that reinforce the angiogenic response (**Figure 1.8**) (Weis & Cheresh 2011). Although the angiogenic activity of a tumor does not necessarily correlate with its aggressiveness, in certain tumor types, it can be a prognostic factor (Bergers & Benjamin 2003).

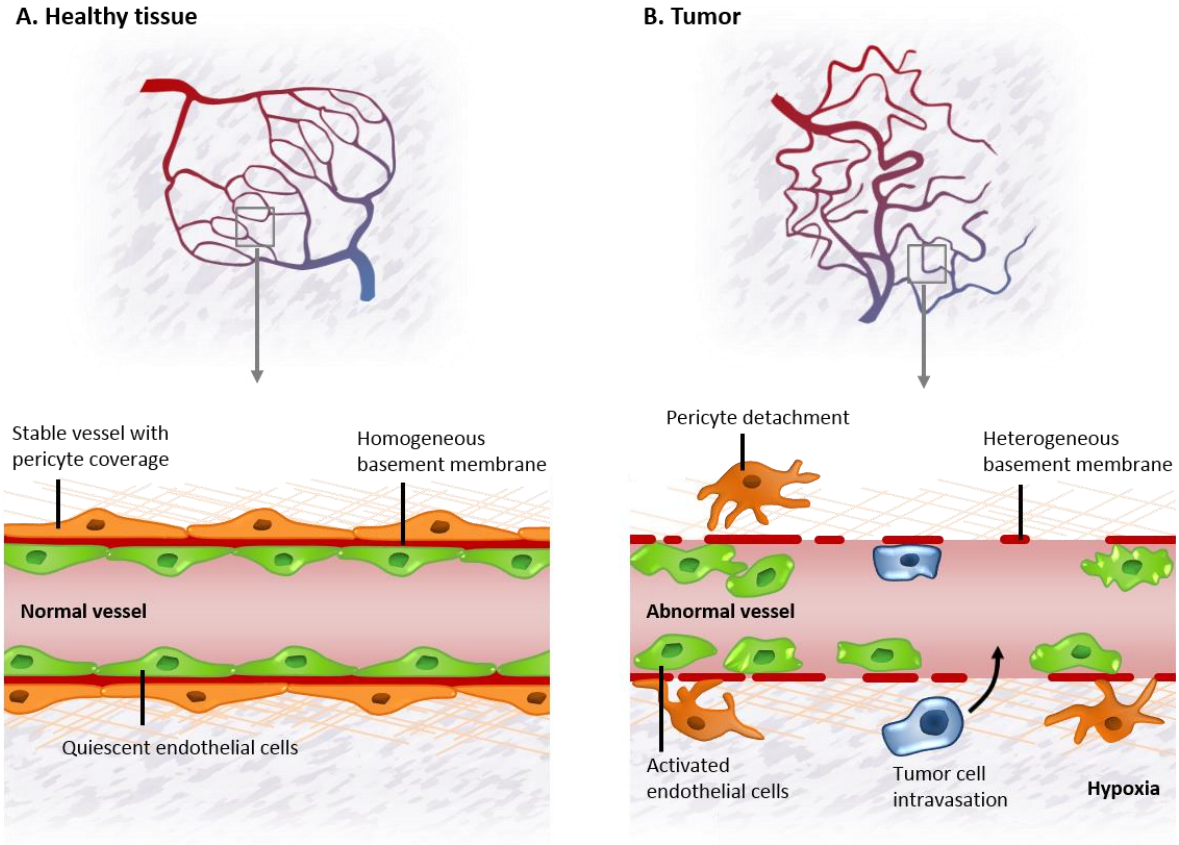

Figure 1.8. Tumor vessels are structurally and functionally abnormal

(A) In healthy tissues, a regularly patterned and functioning vasculature is formed with quiescent endothelial cells and a normal vessel wall, covered by pericytes and smooth muscle cells that maintain vessel integrity. **(B)** In established tumors, the vasculature is chaotic and lacks the hierarchical organization of arteries, capillaries and veins, characteristic of normal vessels. Mural cells detach from the mature blood vessels, compromising their integrity and allowing endothelial cell remodeling and the acquisition of an activated phenotype, which disrupts the vascular barrier and facilitates tumor cell intravasation and metastasis. Cancer stem cells can also differentiate into endothelial cells, or tumor cells can themselves physically participate in the formation of new vessels through vascular mimicry, increasing the angiogenic response. Although the angiogenic response is usually increased in a tumor setting, the newly formed vessels are tortuous and leaky, resulting in elevated levels of hypoxia within the tumor site, which render the tumor cells more invasive and increases their intravasation into blood vessels and metastasis formation in other tissues.

Stromal endothelial cells directly influence tumor progression.

Besides their role in increasing blood flow and nutrient delivery to tumors, endothelial cells also express factors that directly regulate tumor progression and therapeutic resistance. The model stating that endothelial cells could establish an instructive vascular niche that, through the paracrine release of endothelial-derived growth factors and chemokines, termed ‘angiocrine factors’, support tumor growth, was first proposed by

Butler and his colleagues (Butler, Kobayashi, et al. 2010). These factors play a role both in the maintenance of physiological homeostasis and in disease. They comprise pro-angiogenic factors (FGF2 and VEGF), adhesion molecules (ICAM1, VCAM1, E-Selectin and P-Selectin), chemokines involved in leukocyte recruitment (IL8, MCP1 and stromal cell-derived factor 1 [SDF1 or CXCL12]), matrix metalloproteases (MMP10), ECM components (laminin and collagen) and molecules involved in pericyte attachment (PDGFβ), among others (Butler, Kobayashi, et al. 2010).

There have been a number of studies showing that the crosstalk between endothelial and tumor cells regulates cell proliferation and migration. In breast cancer patients, augmented expression of the Ephrin A2 receptor (EphA2) in tumor endothelial cells correlates with poor prognosis because EhpA2 negatively regulates Slit homologue protein 2 (Slit2) which is a breast tumor suppressive angiocrine factor (Brantley-Sieders et al. 2011). VEGF-treated endothelial cells upregulate CXCL1 and CXCL8, which promotes the invasion of CXCR2-positive oral squamous cell carcinoma and Kaposi's sarcoma malignant cells (Warner et al. 2008).

Tumor cells can also induce endothelial cells to synthesize pro-tumoral angiocrine factors. Stimulation of endothelial cells with a combination of factors that mimic several aspects of the tumor microenvironment, increased the endothelial expression of angiocrine pro-inflammatory cytokines, such as MCP1, IL6, IL8, CXCL1, granulocyte colony-stimulating factor (G-CSF or CSF3) and granulocyte macrophage colony-stimulating factor (GM-CSF or CSF2), which increased tumor invasiveness and metastasis (Franses et al. 2013). B cell lymphoma cells produce FGF4 that activates FGFR1 in endothelial cells leading to the upregulation of Jag1. In turn, Jag1 induces Notch2 signaling in lymphoma cells enhancing their invasion and chemoresistance (Cao et al. 2014). The endothelial-tumor cell crosstalk can also regulate other tumor characteristics, such as the stem cell-like properties of cancer cells and the epithelial to mesenchymal transition (EMT) in cancer cells (Lee et al. 2015).

Stromal endothelial cells interact with other tumor stromal components

The contemporary view of cancer envisions tumors as complex systems, consisting not only of tumor cells, but also of diverse collections of recruited stromal cells that regulate tumor cells behavior. In addition to the endothelial cells and pericytes, cancer-associated

fibroblasts (CAFs) and infiltrating cells of the immune system are increasingly accepted to be generic constituents of tumors (**Figure 1.9**) (Franses et al. 2011).

In most epithelial tumors, CAFs constitute the predominant cell population of the tumor stroma. They differ from normal tissue fibroblasts in that they have a higher proliferation rate and enhance tumor phenotypes, such as angiogenesis, cancer cell proliferation, invasion and metastasis. This is achieved by enhanced collagen production, secretion of a wide range of growth factors (hepatocyte growth factor [HGF], insulin-like growth factor [IGF], FGF, VEGF) and other ECM modulators (MMP1), as well as the activation of unique expression programs that facilitate tumor growth (SDF1, interleukin 6 [IL6]) (Madar et al. 2013; Franses et al. 2011). Interestingly, endothelial cells can also contribute to the CAF pool by a process of endothelial to mesenchymal transition (EndoMT) triggered by TGFβ treatment (Zeisberg et al. 2007).

The inflammatory cells that are recruited to the tumor site and become part of the tumor microenvironment can operate in conflicting ways. Both tumor-antagonizing and tumor-promoting leukocytes can be found within a tumor. Recruitment of active immunosuppressive cells, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), can be used by the tumor to suppress the actions of cytotoxic lymphocytes and circumvent immune recognition and attack (Hanahan & Weinberg 2011). Furthermore, cells from the immune system that initially serve as sentinels, can also be modified by the tumor microenvironment and ultimately be used by cancer cells to promote angiogenesis and tumor growth. Circulating monocytes are attracted to the inflammatory signature in the tumor site, where they can rapidly differentiate into mature tumor-associated macrophages. Macrophages can be either classically (M1) and alternatively (M2) activated. *In vivo*, M1-like macrophages are mostly found in inflamed tissues and are activated mainly by IFNγ and LPS, whereas M2-like macrophages regulate the resolution of inflammation and promote tissue healing, and are activated by stimulus like IL4, IL10, IL13 and macrophage colony-stimulating factor (M-CSF or CSF1) (Martinez & Gordon 2014). The complexity of tumor microenvironmental signals is such that classically and alternatively activated macrophages, as well as intermediate states of activation, are all found in tumors, where they exert distinct functions. M1-like macrophages are anti-tumoral whereas M2-like macrophages, the most common type of macrophages in the tumor microenvironment,

have pro-angiogenic and pro-tumoral functions (Sica & Mantovani 2012; Mantovani et al. 2002).

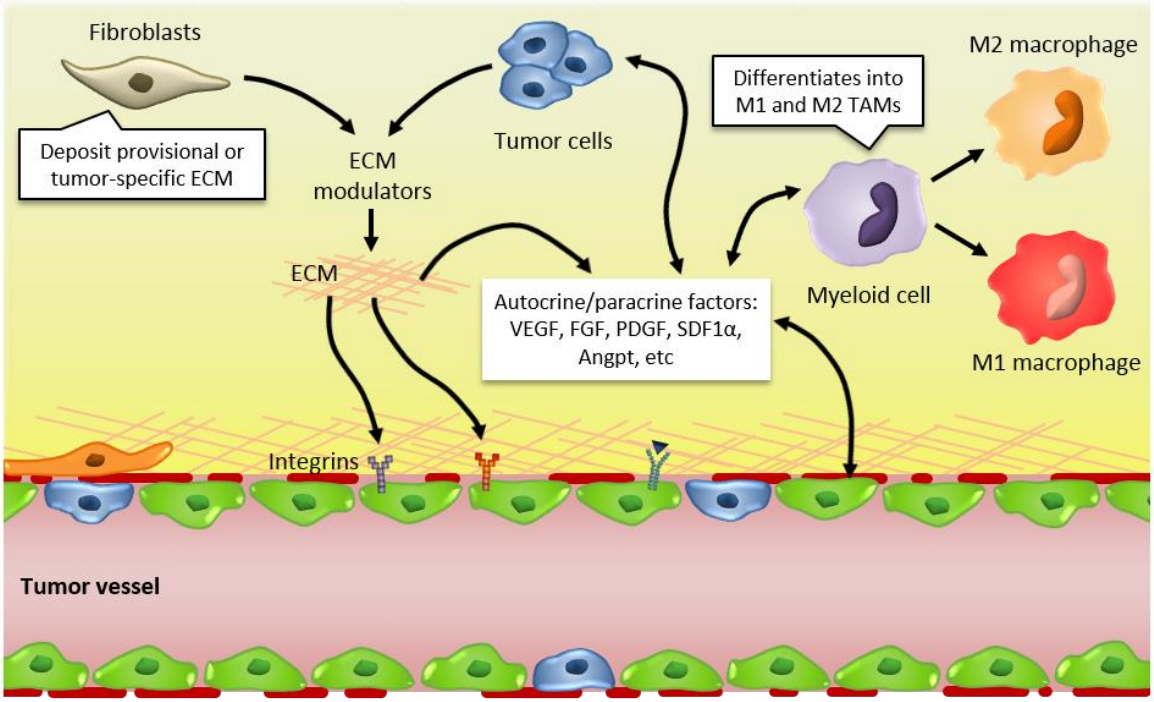

Figure 1.9. Interactions between tumor cells and their microenvironment modulate tumor and angiogenic responses
When tumor size exceeds 1mm^3, pro-angiogenic factors released by the tumor cells induce an angiogenic switch, promoting the rapid growth of new vessels from the preexisting vasculature. These signals also recruit fibroblasts that deposit several ECM proteins and produce ECM remodeling proteins. This promotes the release ECM fragments that affect the function of integrins on neighbor endothelial cells and exposure of cryptic binding sites, inducing blood vessel growth and promoting tumor expansion and progression. Most tumors exhibit an inflammatory response, triggered by the release of a range of soluble factors produced by tumor and endothelial cells that induce the mobilization and homing of myeloid cells from the bone marrow and their differentiation into M1- or M2-like tumor-associated macrophages. The tumor environment is highly dynamic, where endothelial and tumor-derived factors can influence the macrophage polarization and, simultaneously, factors produced by activated macrophages can themselves regulate the activation of signaling pathways that lead to tumor and endothelial cell migration, survival and proliferation.

Tumor-associated macrophages (TAMs) are often found in direct contact with the endothelial cells of uncoated or partially coated blood vessels. Release of Angpt2 by endothelial cells stimulate Tie2-expressing macrophages to establish direct cell contacts with endothelial cells (Baer et al. 2013). The SDF1/CXCR4 axis may also be involved in promoting macrophage-endothelial cell interactions. In fact, CXCR4$^+$ monocytes recruited in response to VEGF are retained in the perivascular niche in response to endothelial-

derived SDF1 (Grunewald et al. 2006). Consistently, TAMs can enhance endothelial cell activation, proliferation and survival through the release of growth factors and inflammatory cytokines. These include pro-angiogenic mediators, such as VEGF, PlGF, FGF2, IL1β, TNFα, and CXCL8, matrix-metalloproteinases and other proteases that release pro-angiogenic growth factors through ECM digestion, and molecules that modulate endothelial cell migration and survival, like semaphorins (Baer et al. 2013). Furthermore, macrophages engage in tight contacts with the tip cells of sprouting blood vessels and bridge them during vessel anastomosis, to form new vascular intersections (Fantin et al. 2010; Tammela et al. 2011).

A large body of evidence indicates that the macrophage-endothelial cell interactions are bidirectional as endothelial cells may also support the differentiation of M2-like macrophages. Indeed, He and colleagues have demonstrated that monocytes can extravasate and gain residence in the vessel wall, were they acquire a M2-like phenotype, in a contact-dependent manner. Although other angiocrine factors are likely to be involved in this process, endothelial-derived CSF1 was shown to be critical for expansion and survival of the macrophage colonies upon transmigration (He et al. 2012). This endothelial-mediated modulation of macrophage activation is another example of the instructive role that endothelial cells have during tumorigenesis. Nonetheless, a greater understanding of the molecular players involved in this process is required.

Accumulating evidences indicate that dysregulated Notch pathway has a critical role in the progression of a number of malignancies, including leukemia, breast cancer, and prostate cancer. High expression levels of several key members of the Notch cascade, such as Notch1 and Jagged1, have been associated with increased progression and metastatic potential, recurrence and poor overall survival (Alcalay & Meani 2003; Santagata et al. 2004; Reedijk et al. 2005), outcomes that are also more frequent when solid tumors have extensive infiltration of TAMs (Leek et al. 1996; Nishie et al. 1999; Koide et al. 2004; Hanada et al. 2000; He et al. 2012; Espinosa et al. 2011; Ohno et al. 2004).

Although the Notch signaling pathway has already been implicated in macrophage polarization (Wang et al. 2010), the role of endothelial-derived Notch signaling has not been addressed in this setting. Given that high levels of endothelial Jag1 expression have been correlated with increased angiogenesis and tumor progression in a mouse model of

prostate cancer, in this Thesis we will employ the same model to explore the role of endothelial-specific Jag1 expression in macrophage recruitment and activation (**Chapter 2**).

1.5. ENDOTHELIAL CELLS HAVE AN INSTRUCTIVE ROLE IN ORGAN REGENERATION

Endothelial cells have the capacity to adapt to the biological demands of each organ, not only regulating vessel morphology, but similarly to what was described in tumorigenesis, through the expression of unique repertoires of trophic factors, known as angiocrine factors. Recently, Nolan and his co-workers have isolated endothelial cells from different vascular beds and clustered them according to their expression of angiocrine factors and cell surface markers. Interestingly, heart and muscle endothelial cells were closely related, whereas endothelial cells from the bone marrow, liver and spleen displayed similar angiocrine gene expression, correlating with the different functions of each organ (Nolan et al. 2014).

Notably, the expression of these tissue-specific angiocrine factors is dynamic, as physiological stress stimulates the expansion of distinct combinations of angiocrine factors throughout the regenerative process. A multitude of studies have shown that endothelial-derived angiocrine genes contribute to tissue regeneration after liver and lung damage and facilitate bone marrow recovery (Ding et al. 2014; Ding et al. 2010; Hu et al. 2014; Ding et al. 2011; Kobayashi et al. 2010; Hooper et al. 2009). Despite the liver's ability to undergo regeneration, chronic or vast injury may often cause liver fibrosis that ultimately promotes cirrhosis and hepatic failure. Liver sinusoidal endothelial cells (LSECs) appear to regulate the delicate balance between the efficient liver regeneration and the inflammatory and fibrotic phenotype, characteristic of chronic liver disease, through the differential expression of SDF1 receptors, CXCR7 and CXCR4. After acute injury, CXCR7 upregulation in LSECs induces hepatocyte proliferation and regeneration. In chronic injury models, however, the CXCR7 pathway is perturbed by constitutive FGFR1 activation that diverts SDF1 signaling in LSECs to a CXCR4-dominated response. This induces a shift in the angiocrine response of LSECs, stimulating the proliferation of hepatic stellate-like cells that

promote liver fibrosis. Interestingly, pharmacological activation of CXCR7 in chronic injury models was able to prevent liver fibrosis (Ding et al. 2014). In another study, LSEC-derived Angpt2 was shown to correspond to the hepatocyte followed by endothelial cell proliferation pattern that occurs after partial hepatectomy. During the early phase of liver regeneration, Angpt2 downregulation leads to reduced TGFβ1 production, enabling hepatocyte proliferation. In the angiogenic phase, however, gradual recovery of Angpt2 expression (Nolan et al. 2014) enabled LSECs proliferation by regulating VEGFR2 in an autocrine manner (Hu et al. 2014).

Similar contributions of endothelial cells were identified in lung regeneration after pneumonectomy. VEGFR2 and FGFR1 expression in pulmonary capillary endothelial cells (PCECs) is essential for MMP14 production by these cells. MMP14 is critical for regenerative alveolarization in the remaining intact lung as it promotes expansion of epithelial progenitor cells by unmasking the epithelial growth factor receptor (EGFR) domains required for its activation (Ding et al. 2011).

Bone marrow endothelial cells have also revealed to be major regulators of hematopoietic recovery following acute injury to the bone marrow microenvironment. Treatment with chemotherapy or irradiation, for example, suppresses hematopoiesis and results in the depletion of hematopoietic stem and progenitor cells (HSPCs), leading to lethal pancytopenias. The interaction of the surviving HSPCs with bone marrow sinusoidal endothelial cells (SECs) regenerates and replenishes the HSPC population after myeloablation, rapidly reconstituting hematopoiesis (Ding et al. 2012; Butler, Kobayashi, et al. 2010; Butler, Nolan, et al. 2010; Doan et al. 2013). Following myeloablation, selective activation of Akt signaling pathway in SECs increases the pool of HSPCs through the upregulation of a specific set of angiocrine factors (Kobayashi et al. 2010). Concurrently, VEGF release upon bone marrow injury stimulates the angiocrine expression of Notch ligands, which prevented the exhaustion of HSPCs (Butler, Nolan, et al. 2010). Endothelial cells can also control the fate of HSPC through the expression of specific micro RNAs. Particularly, endothelial-specific expression of miR-363-5p, which is induced by irradiation, regulates HSPCs proliferation and adhesion to the endothelial cells by modulating specific angiocrine factors, such as stem cell factor (SCF)(Costa et al. 2013).

Recently, Doan and his colleagues have shown that the endothelial cells have a role in protecting mice from injuries to the BM. Following exposure to a lethal dose of total body irradiation, mice with depletion of the pro-apoptotic proteins Bax and Bak in $Tie2^{+}$ endothelial cells demonstrated protection of HSPCs and 100% survival, compared to the depletion of HSPCs and 10% survival of wild-type mice (Doan et al. 2013). Thus, BM SECs are not only able to accelerate reconstitution of mature lineages of hematopoietic cells and simultaneously maintain the stem cell pool, but also to protect HSPCs following acute injuries to the bone marrow.

1.5.1. Regulation of hematopoiesis by the bone marrow niches

It is hypothesized that specific microenvironments may exist within the BM area that contain HSCs and other supporting cells that organize cell-cell interactions and release factors that sustain specific aspects of hematopoiesis, such as HSC survival, self-renewal and differentiation. These processes have been linked to a number of different stromal cell types and signaling pathways (Sugiyama & Nagasawa 2012; He et al. 2014).

The particular areas where HSCs reside and that support hematopoiesis in a stratified manner, where termed “stem-cell niches” by Schofield in 1978 (Schofield 1978). Osteoblasts, the major bone-forming cells, and endothelial cells were the first to arise as important components of these “niches” and suggested to regulate HSCs biology, comprising the osteoblastic and vascular niches, respectively (Calvi et al. 2003; Kiel et al. 2005). Whereas the osteoblastic niche provided a quiescent HSC microenvironment, the vascular niche regulated proliferation, differentiation and mobilization of HSCs (or their committed progenitors) (He et al. 2014). Current data now suggest that the specialized niches that determine hematopoietic stem and progenitor cell fate are far more complex and are created by multiple cell types that contribute to the niches in unique, as well as redundant ways (Ding & Morrison 2013). Similar to endothelial cells and osteoblasts, osteoclasts, adipocytes, reticular cells, neurons and mesenchymal stem cells (MSCs), as well as differentiated cells from the hematopoietic lineage, are now accepted to contribute to regulate HSCs fate and hematopoiesis (**Figure 1.10**) (He et al. 2014).

Perivascular niches dictate HSCs fate

Contrary to what was first thought, several lines of evidence now suggest that osteoblasts may not have a direct effect in hematopoietic stem or progenitor cell maintenance but rather affect the formation or maintenance of HSC niches. In fact, although high numbers of HSCs reside in the trabecular (bone)-rich metaphysis, only few HSCs are in contact with osteoblastic cells. Instead, they are mainly found close to the blood vessels that are adjacent to osteoblasts (Morrison & Scadden 2014; Nombela-Arrieta et al. 2013; Kiel et al. 2009; Sugiyama et al. 2006; Lo Celso et al. 2009). N-Cadherin, proposed to establish homophilic interactions between HSCs and osteoblasts and promote HSC maintenance, has now been shown to have no effect on HSC frequency or function when conditionally deleted from HSCs or from the osteoblast lineage (Bromberg et al. 2012; Kiel et al. 2009). Furthermore, studies that either depleted (Kiel et al. 2007; Visnjic et al. 2004) or increased (Lymperi et al. 2008) osteoblasts had no acute effect on HSC frequency. Instead, inhibition of osteoblastic differentiation through the conditional deletion of *Oxterix* was found to eliminate hematopoiesis in the metaphysis due to the reduction of vessel-covered bone ossicles (Zhou et al. 2010), suggesting a role for mature osteolineage cells in the formation and maintenance of HSC perivascular niches.

The bone marrow is highly vascularized and uniformly occupied by sinusoids. However, the endosteal region is also perfused by arterioles that run along the endosteal area and that further subdivide into arterial capillaries that ultimately connect with sinusoids. Since both sinusoids and arterioles are closely associated with HSCs (Sugiyama et al. 2006; Kunisaki et al. 2013), Frenette has recently suggested that HSC distribution between those niches could represent specific microenvironments that regulated HSC pools differently (**Figure 1.10**) (Boulais & Frenette 2015). In fact, quiescent HSCs were found to associate preferentially with arterioles whereas proliferative HSCs moved away from arterioles and were mainly found in perisinusoidal niches (Kunisaki et al. 2013). The presence of distinct subsets of stromal cells within each vascular niche provided some insights on the mechanism by which HSCs were differentially regulated. Whereas arterioles are ensheathed exclusively by NG2-positive quiescent pericytes, sinusoids are covered by Leptin receptor (LepR)-positive MSCs (Kunisaki et al. 2013). Conditional depletion of $NG2^{+}$ cells induced HSC cycling and reduced long-term repopulating HSCs (LT-HSCs) (Kunisaki et

al. 2013), indicating that functionally distinct vascular niches require the participation of several stromal cell types that differentially affect HSCs fate.

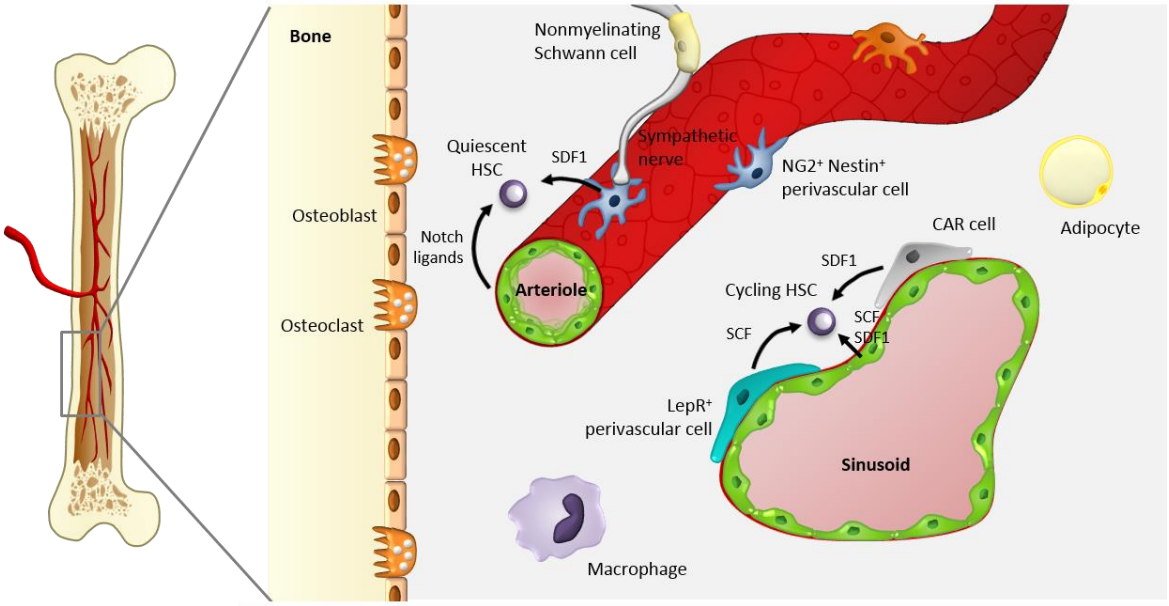

Figure 1.10. The bone marrow HSC niche is composed by distinct stromal cell types and differentiated hematopoietic cells

The vasculature has emerged as a key structure for the maintenance of hematopoietic stem cells (HSCs) in the bone marrow. Throughout the bone marrow (BM), HCSs are mainly found adjacent to sinusoids, where endothelial cells and perivascular mesenchymal stromal cells (MSCs), such as CAR cells and LepR-positive MSCs, produce SCF, SDF1 and other factors that promote HSC proliferation and differentiation. Quiescent HSCs on the other hand, are mostly found around arterioles, where they are in close contact with other types of MSCs, such as Nestin$^+$ NG2 perivascular cells, that release SDF1 and several other factors that promote HSC dormancy. Expression of the Notch ligands Jag1 and Jag2 by endothelial cells also induces HSCs self-renewal. Other cell types that regulate HSC niches include sympathetic nerves, non-myelinating Shwann cells, osteoclasts, adipocytes and differentiated hematopoietic cells, such as macrophages. Osteoblasts do not participate directly in HSC maintenance, but do promote the establishment and maintenance of the HSC perivascular niches.

Endothelial cells regulate HSCs

Endothelial cells also contribute to the perivascular HSC niche. The earliest functional evidence supporting this possibility was the observation that the expression of Glycoprotein 130 (gp130), a signaling subunit shared by IL6 family of cytokines receptors, in endothelial cells significantly contributed to hematopoiesis. Deleting gp130 simultaneously in hematopoietic and endothelial cells induced BM dysfunction, with reduced LT-HSC ability to produce hematopoietic cells. Normal hematopoiesis was restored when gp130-deficient BM was transplanted into irradiated wild-type mice but

hematopoietic defects still existed when wild-type BM was transplanted into irradiated gp130-deficient mice, providing an evidence that endothelial cells contribute to hematopoiesis by responding to signals from the IL6 cytokine family in a gp130-dependent manner (Yao et al. 2005). However, this study did not address whether endothelial cells directly or indirectly regulated HSC maintenance or whether the expression of some angiocrine genes was modified.

Other studies have provided further information on endothelial cell function in hematopoiesis. E-Selectin is exclusively expressed by endothelial cells in the bone marrow and promotes HSC proliferation. Conversely, E-Selectin-deficient mice or mice treated with E-Selectin antagonists show enhanced HSC quiescence and self-renewal potential (Winkler et al. 2010). Disruption of signaling pathways that maintain endothelial cells angiogenic activity, with neutralizing monoclonal antibodies against VE-Cadherin and VEGFR2, also showed that sinusoidal endothelial cells expand the HSC pool, support self-renewal and prevent exhaustion of HSCs both *in vivo* and in serum-free co-culture assays, in a Notch-signaling dependent manner (Butler, Nolan, et al. 2010). Endothelial cells express the Notch ligands Jag1 and Jag2 that support proliferation of Notch-positive HSCs. Endothelial-specific deletion of Jag1 impairs HSC expansion and inhibits hematopoietic recovery following sub-lethal irradiation (Poulos et al. 2013). However, upregulation of angiogenic expression of the Notch ligands shifts the balance from lineage-specific differentiation of LT-HSCs towards expansion, restoring the LT-HSC pool (Butler, Nolan, et al. 2010).

Several other endothelial-derived molecules have been implicated in HSC maintenance. SCF has been suggested to be expressed by endothelial cells, bone marrow fibroblasts, osteoblasts, CAR cells and Nestin-expressing MSCs ($NG2^+$ MSCs) (Ding et al. 2012). SCF has a non-cell-autonomous role for HSC maintenance *in vivo*. Mice expressing a soluble and not membrane-bound SCF isoform (*Sl/Sl*^{d}) are unable to maintain hematopoiesis due to the loss of HSCs (Barker 1994), indicating that membrane-bound SCF is required for HSC maintenance. Analysis of the *Scf* expression pattern has revealed that it is found perivascularly mainly around sinusoids, where $LepR^+$ perivascular stromal cells expressed the highest levels followed by endothelial cells. Conditional deletion of SCF from hematopoietic cells (*Vav-Cre*), osteoblastic cells (*Col2.3-Cre*) and Nestin-expressing perivascular stromal cells (*Nes-Cre* and *Nes-CreER*) did not affect HSC frequency. However,

depleting SCF from perivascular stromal cells (*LepR-Cre*) or endothelial cells (*Tie2-Cre*) depleted HSCs (Ding et al. 2012), demonstrating that there is a perivascular niche for HSCs in which endothelial cells and MSCs promote HSC maintenance through SCF synthesis (**Figure 1.10**).

Another key niche factor required for HSC maintenance and HSC retention in the bone marrow is SDF1, also known as CXCL12 (Sugiyama et al. 2006; Tzeng et al. 2011; Nagasawa 2007). Similar to SCF, SDF1 is also expressed by diverse subsets of bone marrow populations. It is primary expressed by perivascular mesenchymal stromal cells (CXCL12-abundant reticular [CAR] cells and Nestin-, LepR- and Prx1-expressing cells), with 100-fold lower levels in endothelial cells and 1.000-fold lower levels in osteoblasts (Ding & Morrison 2013; Greenbaum et al. 2013). HSC frequency and retention were not affected when SDF1 was conditionally deleted from osteoblasts or their progenitors (*Col2.3-Cre* and *Sp7-Cre*), hematopoietic cells (*Vav-Cre*) or Nestin-positive mesenchymal stromal cells (*Nes-Cre*). SDF1 depletion from perivascular mesenchymal cells using *Prx1-Cre* and *LepR-Cre*, on the other hand, depleted and mobilized HSCs, respectively (Ding & Morrison 2013; Greenbaum et al. 2013). Despite the low levels of SDF1 expression in endothelial cells, HSCs were depleted but not mobilized when this factor was knocked out from endothelial cells (*Tie2-Cre*) (Ding & Morrison 2013; Greenbaum et al. 2013), emphasizing the specific role of endothelial-derived factors in the maintenance of HSCs in the bone marrow.

Hematopoietic progenitors are regulated by distinct BM niches

Much of the focus on bone marrow has been on the HSC niche. However, some subsets of stromal cells also provide a cellular platform for the differentiation of lineage-committed hematopoietic progenitors and thus not only contribute to the maintenance of HSCs but also reconstitute multi-lineage hematopoiesis. For example, conditional deletion of SDF1 in osteoblasts (*Col2.3-Cre*) or perivascular stromal cells (*Prx-Cre*) resulted in the depletion of common lymphoid progenitors (CLPs), whereas its depletion in osteoprogenitors and perivascular CAR cells (*Osx-Cre*) induced a decrease in committed B-lymphoid progenitors (Ding & Morrison 2013; Greenbaum et al. 2013). Pre-pro B cells are found in close contact with CAR cells (Tokoyoda et al. 2004) and consistently, ablation of CAR cells leads to the loss of CLPs and pro-B cells as well as erythroid progenitors (Omatsu et al. 2010). A similar

phenotype was observed after selective elimination of osteoblasts (*Col2.3Δ-TK*), which lead to the depletion of B lymphoid progenitors (Zhu et al. 2007). Therefore, although the osteolineage cells do not directly regulate HSC maintenance they may constitute a suitable microenvironment, distinct from the perivascular niche that maintains HSCs, for the maintenance of lymphoid progenitors.

Bone marrow endothelial cells may also have an instructive role for the differentiation of hematopoietic progenitors. *In vitro*, endothelial-derived SDF1 increases megakaryocyte transendothelial migration and promotes thrombopoietin (Thpo)-independent platelet production (Avecilla et al. 2004; Hamada et al. 1998), suggesting that chemokine-mediated interactions of megakaryocyte progenitors with sinusoidal BM endothelial cells is important for thrombopoiesis *in vitro*. Indeed, SDF1, together with endothelial fibroblast growth factor 4 (FGF4), is able to overcome the thrombocytosis in Thpo- and Thpo receptor (c-Mpl)-deficient mice (Avecilla et al. 2004) by inducing the expression of adhesion molecules in both megakaryocytes (VLA4) and BM endothelial cells (VCAM1) (Avraham et al. 1993; Avraham et al. 1994).

1.6. AIMS OF THE THESIS

Endothelial cells are crucial players in a variety of processes, such as tumor progression and hematopoiesis. Therefore, targeting specific pathways in endothelial cells often perturbs those events.

In this Thesis, we aimed to scrutinize the role of endothelial derived Notch ligands in the modulation of the BM microenvironment and in tumor development, through three different approaches.

1. To characterize the role of endothelial Jag1 in macrophage recruitment and activation in prostate tumors (**Chapter 2**).

2. To characterize the role of endothelial Dll4 in the crosstalk between BM endothelial cells and hematopoietic cells (**Chapter 3**).

3. To analyze the effects of modifying endothelial Dll4 levels in the maintenance of the BM vascular niche following myeloablation (**Chapter 4**).

1.7. REFERENCES

Abraham, S. et al., 2015. A Rac/Cdc42 exchange factor complex promotes formation of lateral filopodia and blood vessel lumen morphogenesis. *Nature Communications*, 6, p.7286.

Adams, R.H. & Alitalo, K., 2007. Molecular regulation of angiogenesis and lymphangiogenesis. *Nature Reviews Molecular Cell Biology*, 8(6), pp.464–478.

Aird, W.C., 2011. Discovery of the cardiovascular system: From Galen to William Harvey. *Journal of Thrombosis and Haemostasis*, 9, pp.118–129.

Aird, W.C., 2006. Mechanisms of Endothelial Cell Heterogeneity in Health and Disease. *Circulation Research*, 98(2), pp.159–162.

Ajmone-Cat, M.A. et al., 2010. Non-steroidal anti-inflammatory drugs and brain inflammation: Effects on microglial functions. *Pharmaceuticals*, 3, pp.1949–1964.

Alcalay, M. & Meani, N., 2003. Acute myeloid leukemia fusion proteins deregulate genes involved in stem cell maintenance and DNA repair. *Journal of Clinical …*, 112(11).

Alexander, J.S., 2000. Rho, Tyrosine Kinase, Ca2+, and Junctions in Endothelial Hyperpermeability. *Circulation Research*, 87(4), pp.268–271.

Andersson, E.R., Sandberg, R. & Lendahl, U., 2011. Notch signaling: simplicity in design, versatility in function. *Development*, 138(17), pp.3593–3612.

Androutsos, G., Karamanou, M. & Stefanadis, C., 2012. William Harvey (1578-1657): Discoverer of Blood Circulation. *Hellenic Journal of Cardiology*, (53), pp.6–9.

Armulik, A., 2005. Endothelial/Pericyte Interactions. *Circulation Research*, 97(6), pp.512–523.

Armulik, A., Genové, G. & Betsholtz, C., 2011. Pericytes: Developmental, Physiological, and Pathological Perspectives, Problems, and Promises. *Developmental Cell*, 21(2), pp.193–215.

Asahara, T. et al., 1999. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circulation research*, 85(3), pp.221–228.

Aspalter, I.M. et al., 2015. Alk1 and Alk5 inhibition by Nrp1 controls vascular sprouting downstream of Notch. *Nature Communications*, 6, p.7264.

Avecilla, S.T. et al., 2004. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nature medicine*, 10(1), pp.64–71.

Avraham, H. et al., 1993. Characterization of adhesive interactions between human endothelial cells and megakaryocytes. *The Journal of clinical investigation*, 91(6), pp.2378–84.

Avraham, H. et al., 1994. Modulation of megakaryocytopoiesis by human basic fibroblast growth factor. *Blood*, 83(8), pp.2126–32.

Baer, C. et al., 2013. Reciprocal interactions between endothelial cells and macrophages in angiogenic vascular niches. *Experimental Cell Research*, 319(11), pp.1626–1634.

Baluk, P., Hashizume, H. & McDonald, D.M., 2005. Cellular abnormalities of blood vessels as targets in cancer. *Current Opinion in Genetics & Development*, 15(1), pp.102–111.

Barker, J.E., 1994. Sl/Sld hematopoietic progenitors are deficient in situ. *Experimental hematology*, 22(2), pp.174–7.

Bazzoni, G. et al., 2004. Endothelial Cell-to-Cell Junctions : Molecular Organization and Role in Vascular Homeostasis. , pp.869–901.

Belton, O. & Fitzgerald, D., 2003. Cyclooxygenase-2 Inhibitors and Atherosclerosis. *Journal of the American College of Cardiology*, 41(10), pp.1838–1839.

Benedito, R. et al., 2009. The Notch Ligands Dll4 and Jagged1 Have Opposing Effects on Angiogenesis ¨. *Cell*, 137, pp.1124–1135.

Bergers, G. & Benjamin, L.E., 2003. Angiogenesis: Tumorigenesis and the angiogenic switch. *Nature Reviews Cancer*, 3(6), pp.401–410.

Bergers, G. & Song, S., 2005. The role of pericytes in blood-vessel formation and maintenance. *Neuro-oncology*, 7(4), pp.452–64.

Bernatchez, P.N. et al., 2002. Relative effects of VEGF-A and VEGF-C on endothelial cell proliferation, migration and PAF synthesis: Role of neuropilin-1. *Journal of cellular biochemistry*, 85(3), pp.629–39.

Berra, E. et al., 2000. Signaling angiogenesis via p42/p44 MAP kinase and hypoxia. *Biochemical Pharmacology*, 60(8), pp.1171–1178.

Bevilacqua, M.P. et al., 1986. Recombinant tumor necrosis factor induces procoagulant activity in cultured human vascular endothelium: characterization and comparison with the actions of interleukin 1. *Proceedings of the National Academy of Sciences of the United States of America*, 83(12), pp.4533–7.

Bilsborough, W. et al., 2006. Anti-tumour necrosis factor-alpha therapy over conventional therapy improves endothelial function in adults with rheumatoid arthritis. *Rheumatology International*, 26(12), pp.1125–1131.

Blanco, R. & Gerhardt, H., 2013. VEGF and Notch in tip and stalk cell selection. *Cold Spring Harbor perspectives in medicine*, 3(1), p.a006569.

De Bock, K. et al., 2009. Endothelial oxygen sensors regulate tumor vessel abnormalization by instructing phalanx endothelial cells. *Journal of molecular medicine*, 87(6), pp.561–569.

Boulais, P.E. & Frenette, P.S., 2015. Making sense of hematopoietic stem cell niches. *Blood*, 125(17), pp.2621–9.

Braga, V.M.M., 2002. Cell-cell adhesion and signalling. *Current Opinion in Cell Biology*, 14, pp.546–556.

Brantley-Sieders, D.M. et al., 2011. Angiocrine factors modulate tumor proliferation and motility through EphA2 repression of Slit2 tumor suppressor function in endothelium. *Cancer research*, 71(3), pp.976–87.

Bray, S.J., 2006. Notch signalling: a simple pathway becomes complex. *Nature reviews. Molecular cell biology*, 7(9), pp.678–89.

Brindle, N.P.J., 2006. Signaling and Functions of Angiopoietin-1 in Vascular Protection. *Circulation Research*, 98(8), pp.1014–1023.

Broermann, A. et al., 2011. Dissociation of VE-PTP from VE-cadherin is required for leukocyte extravasation and for VEGF-induced vascular permeability in vivo. *Journal of Experimental Medicine*, 208(12), pp.2393–2401.

Bromberg, O. et al., 2012. Osteoblastic N-cadherin is not required for microenvironmental support and regulation of hematopoietic stem and progenitor cells. *Blood*, 120(2), pp.303–13.

Burri, P.H., Hlushchuk, R. & Djonov, V., 2004. Intussusceptive angiogenesis: Its emergence, its characteristics, and its significance. *Developmental Dynamics*, 231(3), pp.474–488.

Butler, J.M., Nolan, D.J., et al., 2010. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell*, 6(3), pp.251–264.

Butler, J.M., Kobayashi, H., et al., 2010. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nature reviews. Cancer*, 10(2), pp.138–146.

Caduff, J.H., Fischer, L.C. & Burri, P.H., 1986. Scanning electron microscope study of the developing microvasculature in the postnatal rat lung. *The Anatomical record*, 216(2), pp.154–164.

Calvi, L.M. et al., 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*, 425(6960), pp.841–846.

Cao, N. & Yao, Z.X., 2011. The Hemangioblast: From Concept to Authentication. *Anatomical Record*, 294(4), pp.580–588.

Cao, Z. et al., 2014. Angiocrine Factors Deployed by Tumor Vascular Niche Induce B Cell Lymphoma Invasiveness and Chemoresistance. *Cancer Cell*, 25(3), pp.350–365.

Carlson, T.R. et al., 2005. Endothelial expression of constitutively active Notch4 elicits reversible arteriovenous malformations in adult mice. *Proceedings of the National Academy of Sciences of the United States of America*, 102(28), pp.9884–9.

Carmeliet, P. et al., 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*, 380(6573), pp.435–439.

Castermans, K. & Griffioen, A.W., 2007. Tumor blood vessels, a difficult hurdle for infiltrating leukocytes. *Biochimica et Biophysica Acta (BBA) - Reviews on Cancer*, 1776(2), pp.160–174.

Lo Celso, C. et al., 2009. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature*, 457(7225), pp.92–96.

Chen, S. & Lechleider, R.J., 2004. Transforming Growth Factor-β–Induced Differentiation of Smooth Muscle From a Neural Crest Stem Cell Line. *Circulation Research*, pp.1195–1202.

Choi, K. et al., 1998. A common precursor for hematopoietic and endothelial cells. *Development (Cambridge, England)*, 125(4), pp.725–732.

Cines, D.B. et al., 1998. Endothelial Cells in Physiology and in the Pathophysiology of Vascular Disorders. *Blood*, 91(10), pp.3527–3561.

Claxton, S. & Fruttiger, M., 2004. Periodic Delta-like 4 expression in developing retinal arteries. *Gene expression patterns : GEP*, 5(1), pp.123–7.

Conway, E.M., Collen, D. & C, P.C., 2001. Molecular mechanisms of blood vessel growth. *Cardiovascular Research*, 49, pp.507–521.

Costa, A. et al., 2013. miR-363-5p regulates endothelial cell properties and their communication with hematopoietic precursor cells. *Journal of hematology & oncology*, 6(1), p.87.

Coultas, L., Chawengsaksophak, K. & Rossant, J., 2005. Endothelial cells and VEGF in vascular development. *Nature*, 438(7070), pp.937–945.

Crosby, C. V et al., 2005. VE-cadherin is not required for the formation of nascent blood vessels but acts to prevent their disassembly. *Blood*, 105(0006–4971 (Print)), pp.2771–2776.

Danese, S., Dejana, E. & Fiocchi, C., 2007. Immune Regulation by Microvascular Endothelial Cells: Directing Innate and Adaptive Immunity, Coagulation, and Inflammation. *The Journal of Immunology*, 178(10), pp.6017–6022.

Davis, G.E. & Senger, D.R., 2005. Endothelial Extracellular Matrix: Biosynthesis, Remodeling, and Functions During Vascular Morphogenesis and Neovessel Stabilization. *Circulation Research*, 97(11), pp.1093–1107.

Davis, S. et al., 1996. Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell*, 87(7), pp.1161–1169.

Dejana, E., 1996. Endothelial adherens junctions: implications in the control of vascular permeability and angiogenesis. *The Journal of clinical investigation*, 98(9), pp.1949–1953.

Dejana, E., 2004. Endothelial cell-cell junctions: happy together. *Nature Reviews*, 5(April).

Dejana, E., Orsenigo, F. & Lampugnani, M.G., 2008. The role of adherens junctions and VE-cadherin in the control of vascular permeability. *Journal of cell science*, 121(Pt 13), pp.2115–2122.

Dejana, E., Tournier-Lasserve, E. & Weinstein, B.M., 2009. The Control of Vascular Integrity by Endothelial Cell Junctions: Molecular Basis and Pathological Implications. *Developmental cell*, 16(2), pp.209–221.

Diaz-Flores, L. et al., 1991. Microvascular pericytes: A review of their morphological and functional characteristics. *Histology and Histopathology*, 6, pp.269–286.

Díaz-Flores, L. et al., 2009. Pericytes. Morphofunction, interactions and pathology in a quiescent and activated mesenchymal cell niche. *Histology and Histopathology*, 24(7), pp.909–969.

Ding, B.-S. et al., 2014. Divergent angiocrine signals from vascular niche balance liver regeneration and fibrosis. *Nature*, 505(7481), pp.97–102.

Ding, B.-S. et al., 2011. Endothelial-Derived Angiocrine Signals Induce and Sustain Regenerative Lung Alveolarization. *Cell*, 147(3), pp.539–553.

Ding, B.-S. et al., 2010. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature*, 468(7321), pp.310–315.

Ding, L. et al., 2012. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*, 481(7382), pp.457–62.

Ding, L. & Morrison, S.J., 2013. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature*, 495(7440), pp.231–5.

Dixelius, J. et al., 2003. Ligand-induced Vascular Endothelial Growth Factor Receptor-3 (VEGFR-3) Heterodimerization with VEGFR-2 in Primary Lymphatic Endothelial Cells Regulates Tyrosine Phosphorylation Sites. *Journal of Biological Chemistry*, 278(42), pp.40973–40979.

Djonov, V. et al., 2000. Intussusceptive Angiogenesis. , pp.286–293.

Doan, P.L. et al., 2013. Tie2(+) bone marrow endothelial cells regulate hematopoietic stem cell regeneration following radiation injury. *Stem cells (Dayton, Ohio)*, 31(2), pp.327–37.

Domenga, V. et al., 2004. Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. *Genes and Development*, 18(22), pp.2730–2735.

Donovan, J. et al., 2002. Tetralogy of fallot and other congenital heart defects in Hey2 mutant mice. *Curr Biol*, 12(18), pp.1605–1610.

Donoviel, D.B. et al., 1999. Mice lacking both presenilin genes exhibit early embryonic patterning defects. *Genes & development*, 13(21), pp.2801–10.

Drake, C.J., 2003. Embryonic and adult vasculogenesis. *Birth Defects Research Part C: Embryo Today: Reviews*, 69(1), pp.73–82.

Duarte, A. et al., 2004. Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes and Development*, 18(20), pp.2474–2478.

Dumont, D.J. et al., 1998. Cardiovascular failure in mouse embryos deficient in VEGF receptor-3. *Science (New York, N.Y.)*, 282(October), pp.946–949.

Dumont, D.J. et al., 1994. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes and Development*, 8(16), pp.1897–1909.

Ebnet, K. et al., 2004. Junctional adhesion molecules (JAMs): more molecules with dual functions? *Journal of cell science*, 117, pp.19–29.

Eliseyeva, M.R., 2013. Endothelium: A Long Road from Mystery to Discovery. *International Journal of Biomedicine*, 3(1), pp.9–11.

Espinosa, I. et al., 2011. CSF1 expression in nongynecological leiomyosarcoma is associated with increased tumor angiogenesis. *American Journal of Pathology*, 179(4), pp.2100–2107.

Fantin, A. et al., 2013. NRP1 acts cell autonomously in endothelium to promote tip cell function during sprouting angiogenesis. *Blood*, 121(12), pp.2352–2362.

Fantin, A. et al., 2010. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood*, 116(5), pp.829–840.

Felcht, M. et al., 2012. Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signaling. *Journal of Clinical Investigation*, 122(6), pp.1991–2005.

Ferrara, N. et al., 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*, 380(6573), pp.439–442.

Ferrara, N., 2001. Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol*, 280(6), pp.C1358-1366.

Fischer, A. et al., 2004. The Notch target genes Hey1 and Hey2 are required for embryonic vascular development. *Genes & development*, 18(8), pp.901–11.

Folkman, J. et al., 1989. Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature*, 399, pp.58–61.

Folkman, J., 1971. Tumor angiogenesis: therapeutic implications. *The New England Journal of Medicine*.

Fong, G.-H. et al., 1995. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*, 376(6535), pp.66–70.

Franses, J.W. et al., 2013. Dysfunctional endothelial cells directly stimulate cancer inflammation and metastasis. *Int J Cancer*, 18(9), pp.1199–1216.

Franses, J.W. et al., 2011. Stromal endothelial cells directly influence cancer progression. *Science Translational Medicine*, 3(66).

Fujii, T. & Kuwano, H., 2010. Regulation of the expression balance of angiopoietin-1 and angiopoietin-2 by Shh and FGF-2. *In vitro cellular & developmental biology. Animal*, 46(6), pp.487–91.

Funahashi, Y. et al., 2010. Notch regulates the angiogenic response via induction of VEGFR-1. *Journal of angiogenesis research*, 2, p.3.

Gale, N.W. et al., 2002. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. *Developmental Cell*, 3(3), pp.411–423.

Gale, N.W. et al., 2004. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proceedings of the National Academy of Sciences of the United States of America*, 101(45), pp.15949–15954.

Galley, H.F., 2004. Physiology of the endothelium. *British Journal of Anaesthesia*, 93(1), pp.105–113.

Garber, A., Livingston, M. & McAlister, V., 2008. History of the Circulatory System : discovery of the basics. *Uwomj*, 77(2), pp.22–24.

Garlanda, C. & Dejana, E., 1997. Heterogeneity of Endothelial Cells : Specific Markers. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 17(7), pp.1193–1202.

Gavard, J., 2009. Breaking the VE-cadherin bonds. *FEBS Letters*, 583(1), pp.1–6.

Gavard, J., Patel, V. & Gutkind, J.S., 2008. Angiopoietin-1 prevents VEGF-induced endothelial permeability by sequestering Src through mDia. *Developmental cell*, 14(1), pp.25–36.

Gerhardt, H. et al., 2004. Neuropilin-1 is required for endothelial tip cell guidance in the developing central nervous system. *Developmental Dynamics*, 231(3), pp.503–509.

Gerhardt, H. et al., 2003. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *The Journal of Cell Biology*, 161(6), pp.1163–77.

Gerhardt, H. & Betsholtz, C., 2003. Endothelial-pericyte interactions in angiogenesis. *Cell and Tissue Research*, 314(1), pp.15–23.

Gessler, M. et al., 2002. Mouse gridlock: no aortic coarctation or deficiency, but fatal cardiac defects in Hey2 -/- mice. *Current biology : CB*, 12(18), pp.1601–4.

Geudens, I. & Gerhardt, H., 2011. Coordinating cell behaviour during blood vessel formation. , 4583, pp.4569–4583.

Ghosh, K. et al., 2008. Tumor-derived endothelial cells exhibit aberrant Rho-mediated mechanosensing and abnormal angiogenesis in vitro. *Proceedings of the National Academy of Sciences of the United States of America*, 105(32), pp.11305–11310.

Giannotta, M., Trani, M. & Dejana, E., 2013. VE-cadherin and endothelial adherens junctions: Active guardians of vascular integrity. *Developmental Cell*, 26(5), pp.441–454.

Glomski, K. et al., 2011. Deletion of Adam10 in endothelial cells leads to defects in organ-specific vascular structures. *Blood*, 118(4), pp.1163–1174.

González-Mariscal, L., Tapia, R. & Chamorro, D., 2008. Crosstalk of tight junction components with signaling pathways. *Biochimica et Biophysica Acta - Biomembranes*, 1778(3), pp.729–756.

Gory-Fauré, S. et al., 1999. Role of vascular endothelial-cadherin in vascular morphogenesis. *Development (Cambridge, England)*, 126, pp.2093–2102.

Grammas, P., 2011. Neurovascular dysfunction, inflammation and endothelial activation: implications for the pathogenesis of Alzheimer's disease. *Journal of neuroinflammation*, 8(1), p.26.

Greenbaum, A. et al., 2013. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature*, 495(7440), pp.227–30.

Grunewald, M. et al., 2006. VEGF-induced adult neovascularization: Recruitment, retention, and role of accessory cells. *Cell*, 124(1), pp.175–189.

Gu, C. et al., 2003. Neuropilin-1 Conveys Semaphorin and VEGF Signaling during Neural and Cardiovascular Development. *Developmental Cell*, 5(1), pp.45–57.

Gullestad, L. & Aukrust, P., 2005. Review of Trials in Chronic Heart Failure Showing Broad-Spectrum Anti-Inflammatory Approaches. *The American Journal of Cardiology*, 95(11), pp.17–23.

Gupta, K. et al., 1999. VEGF prevents apoptosis of human microvascular endothelial cells via opposing effects on MAPK/ERK and SAPK/JNK signaling. *Experimental cell research*, 247(2), pp.495–504.

Hadland, B.K. et al., 2004. A requirement for Notch1 distinguishes 2 phases of definitive hematopoiesis during development. *Blood*, 104(10), pp.3097–3105.

Hamada, B.T. et al., 1998. Transendothelial Migration of Megakaryocytes in Response to Stromal Cell-derived Factor 1 (SDF-1) Enhances Platelet Formation. , 188(3).

Hamada, Y. et al., 1999. Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. *Development (Cambridge, England)*, 126(15), pp.3415–3424.

Hanada, T. et al., 2000. Prognostic value of tumor-associated macrophage count in human bladder cancer. *International Journal of Urology*, 7(7), pp.263–269.

Hanahan, D. & Weinberg, R.A., 2011. Review Hallmarks of Cancer : The Next Generation. *Cell*, 144(5), pp.646–674.

Harrington, L.S. et al., 2008. Regulation of multiple angiogenic pathways by Dll4 and Notch in human umbilical vein endothelial cells. *Microvascular Research*, 75(2), pp.144–154.

Hartmann, D. et al., 2002. The disintegrin/metalloprotease ADAM 10 is essential for Notch signalling but not for alpha-secretase activity in fibroblasts. *Human molecular genetics*, 11(21), pp.2615–2624.

Hatanaka, K. et al., 2012. Fibroblast growth factor signaling potentiates VE-cadherin stability at adherens junctions by regulating SHP2. *PLoS ONE*, 7(5), pp.1–10.

Hayashi, M. et al., 2013. VE-PTP regulates VEGFR2 activity in stalk cells to establish endothelial cell polarity and lumen formation. *Nature Communications*, 4, p.1672.

He, H. et al., 2012. Endothelial cells provide an instructive niche for the differentiation and functional polarization of M2-like macrophages. *Blood*, 120(15), pp.3152–3162.

He, N. et al., 2014. Bone marrow vascular niche: home for hematopoietic stem cells. *Bone marrow research*, 2014, p.128436.

Heidemann, J., 2003. Angiogenic Effects of Interleukin 8 (CXCL8) in Human Intestinal Microvascular Endothelial Cells Are Mediated by CXCR2. *Journal of Biological Chemistry*, 278(10), pp.8508–8515.

Hellström, M. et al., 2007. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature*, 445(7129), pp.776–780.

Hellström, M. et al., 1999. Role of PDGF-B and PDGFR-beta in recruitment of vascular smooth muscle cells and pericytes during embryonic blood vessel formation in the mouse. *Development (Cambridge, England)*, 126, pp.3047–3055.

Heltianu, C., Simionescu, M. & Simionescu, N., 1982. Histamine receptors of the microvascular endothelium revealed in situ with a histamine-ferritin conjugate: Characteristic high-affinity binding sites in venules. *Journal of Cell Biology*, 93, pp.357–364.

Henderson, R.B. et al., 2003. Rapid recruitment of inflammatory monocytes is independent of neutrophil migration. *Blood*, 102(1), pp.328–335.

Herbert, S.P. & Stainier, D.Y.R., 2011. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nature Reviews Molecular Cell Biology*, 12(9), pp.551–564.

Herreman, a et al., 1999. Presenilin 2 deficiency causes a mild pulmonary phenotype and no changes in amyloid precursor protein processing but enhances the embryonic lethal phenotype of presenilin 1 deficiency. *Proceedings of the National Academy of Sciences of the United States of America*, 96(21), pp.11872–11877.

High, F.A. et al., 2008. Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. *Proceedings of the National Academy of Sciences of the United States of America*, 105, pp.1955–1959.

Van Hinsbergh, V.W.M., 2012. Endothelium - Role in regulation of coagulation and inflammation. *Seminars in Immunopathology*, 34(1), pp.93–106.

Hirase, T. et al., 1997. Occludin as a possible determinant of tight junction permeability in endothelial cells. *Journal of cell science*, 110 (Pt 1, pp.1603–1613.

Hiratsuka, S. et al., 1998. Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proceedings of the National Academy of Sciences of the United States of America*, 95(16), pp.9349–9354.

Hirschi, K.K., 2003. Gap Junction Communication Mediates Transforming Growth Factor- Activation and Endothelial-Induced Mural Cell Differentiation. *Circulation Research*, 93(5), pp.429–437.

Hoeben, A. et al., 2004. Vascular endothelial growth factor and angiogenesis. *Pharmacological reviews*, 56(4), pp.549–580.

Hofmann, J.J. & Iruela-Arispe, M.L., 2007. Notch Expression Patterns in the Retina: An Eye on Receptor-Ligand Distribution during Angiogenesis. *Gene expression patterns : GEP*, 7(4), pp.461–470.

Holderfield, M.T. & Hughes, C.C.W., 2008. Crosstalk Between Vascular Endothelial Growth Factor, Notch, and Transforming Growth Factor- β in Vascular Morphogenesis. *Circulation Research*, 102, pp.637–652.

Hooper, A.T. et al., 2009. Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2 mediated regeneration of sinusoidal endothelial cells. *Cell stem cell*, 4(3), pp.263–74.

Hu, B. & Cheng, S.-Y., 2009. Angiopoietin-2: development of inhibitors for cancer therapy. *Current oncology reports*, 11(2), pp.111–116.

Hu, J. et al., 2014. Endothelial Cell-Derived Angiopoietin-2 Controls Liver Regeneration as a Spatiotemporal Rheostat. *Science*, 343(January), pp.416–419.

Huang, H. et al., 2010. Targeting the ANGPT–TIE2 pathway in malignancy. *Nature Reviews Cancer*, 10(8), pp.575–585.

Iruela-Arispe, M.L. & Davis, G.E., 2009. Cellular and Molecular Mechanisms of Vascular Lumen Formation. *Developmental Cell*, 16(2), pp.222–231.

Iso, T. et al., 2006. Dll4-selective Notch signaling induces ephrinB2 gene expression in endothelial cells. *Biochemical and Biophysical Research Communications*, 341(3), pp.708–714.

Itoh, N. & Ornitz, D.M., 2004. Evolution of the Fgf and Fgfr gene families. *Trends in genetics : TIG*, 20(11), pp.563–9.

Jakobsson, L. et al., 2010. Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. *Nature cell biology*, 12(10), pp.943–953.

Jones, E. a V et al., 2008. Separating genetic and hemodynamic defects in neuropilin 1 knockout embryos. *Development (Cambridge, England)*, 135(14), pp.2479–2488.

Kappas, N.C. et al., 2008. The VEGF receptor Flt-1 spatially modulates Flk-1 signaling and blood vessel branching. *The Journal of cell biology*, 181(5), pp.847–58.

Karar, J. & Maity, A., 2011. PI3K/AKT/mTOR Pathway in Angiogenesis. *Frontiers in Molecular Neuroscience*, 4(December), pp.1–8.

Kawasaki, T. et al., 1999. A requirement for neuropilin-1 in embryonic vessel formation. *Development (Cambridge, England)*, 126(21), pp.4895–4902.

Kay, J. & Calabrese, L., 2009. The role of interleukin-1 in the pathogenesis of rheumatoid arthritis. *Rheumatology*, 11(5), pp.365–370.

Khan, I. a, Daya, S.K. & Gowda, R.M., 2005. Evolution of the theory of circulation. *International journal of cardiology*, 98(3), pp.519–21.

Kiel, M.J. et al., 2009. Hematopoietic Stem Cells Do Not Depend on N-Cadherin to Regulate Their Maintenance. *Cell Stem Cell*, 4(2), pp.170–179.

Kiel, M.J. et al., 2005. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*, 121(7), pp.1109–21.

Kiel, M.J., Radice, G.L. & Morrison, S.J., 2007. Lack of Evidence that Hematopoietic Stem Cells Depend on N-Cadherin-Mediated Adhesion to Osteoblasts for Their Maintenance. *Cell Stem Cell*, 1(2), pp.204–217.

Kobayashi, H. et al., 2010. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. , 12(11), pp.1046–1056.

Koide, N. et al., 2004. Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus. *The American journal of gastroenterology*, 99(9), pp.1667–1674.

Kokubo, H. et al., 2005. Mouse hesr1 and hesr2 genes are redundantly required to mediate Notch signaling in the developing cardiovascular system. *Developmental Biology*, 278, pp.301–309.

Konttinen, Y.T. et al., 1998. New collagenolytic enzymes/cascade identified at the pannus-hard tissue junction in rheumatoid arthritis: destruction from above. *Matrix Biol*, 17(8–9), pp.585–601.

Kopan, R. & Ilagan, M.X.G., 2009. The Canonical Notch Signaling Pathway: Unfolding the Activation Mechanism. *Cell*, 137(2), pp.216–233.

Krebs, L.T. et al., 2003. Characterization of Notch3-Deficient Mice: Normal Embryonic Development and Absence of Genetic Interactions with a Notch1 Mutation. *Genesis*, 37, pp.139–143.

Krebs, L.T. et al., 2004. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants service Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. , (207), pp.2469–2473.

Krebs, L.T. et al., 2000. Notch signaling is essential for vascular morphogenesis in mice. *Genes and Development*, 14(11), pp.1343–1352.

Kumano, K. et al., 2003. Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity*, 18(5), pp.699–711.

Kunisaki, Y. et al., 2013. Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature*, 502(7473), pp.637–643.

Kwei, S. et al., 2004. Early Adaptive Responses of the Vascular Wall during Venous Arterialization in Mice. *The American Journal of Pathology*, 164(1), pp.81–89.

Lamalice, L. et al., 2004. Phosphorylation of tyrosine 1214 on VEGFR2 is required for VEGF-induced activation of Cdc42 upstream of SAPK2/p38. *Oncogene*, 23(2), pp.434–445.

Lamalice, L., Le Boeuf, F. & Huot, J., 2007. Endothelial Cell Migration During Angiogenesis. *Circulation Research*, 100(6), pp.782–794.

Lammert, E. & Axnick, J., 2012. Vascular lumen formation. *Cold Spring Harbor perspectives in medicine*, 2(4), p.a006619.

Lee, E., Pandey, N.B. & Popel, A.S., 2015. Crosstalk between cancer cells and blood endothelial and lymphatic endothelial cells in tumour and organ microenvironment. *Expert Rev Mol Med*, 17, p.e3.

Leek, R.D. et al., 1996. Association of Macrophage Infiltration with Angiogenesis and Prognosis in Invasive Breast Carcinoma. *Cancer Research*, 56(16), pp.4625–4629.

Lenard, A. et al., 2013. In vivo analysis reveals a highly stereotypic morphogenetic pathway of vascular anastomosis. *Developmental Cell*, 25(5), pp.492–506.

Leslie, J.D. et al., 2007. Endothelial signalling by the Notch ligand Delta-like 4 restricts angiogenesis. *Development*, 134(5), pp.839–844.

Levéen, P. et al., 1994. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. *Genes and Development*, 8(16), pp.1875–1887.

Libby, P., Ridker, P.M. & Hansson, G.K., 2011. Progress and challenges in translating the biology of atherosclerosis. *Nature*, 473(7347), pp.317–25.

Limbourg, F.P., 2005. Essential Role of Endothelial Notch1 in Angiogenesis. *Circulation*, 111(14), pp.1826–1832.

Lindahl, P. et al., 1997. Pericyte loss and microaneurysm formation in PDGF-B-deficient mice. *Science (New York, N.Y.)*, 277(5323), pp.242–245.

Lindblom, P. et al., 2003. Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. *Genes & development*, 17(15), pp.1835–40.

Liu, H. et al., 2010. Notch3 is critical for proper angiogenesis and mural cell investment. *Circulation research*, 107(7), pp.860–70.

Liu, J. et al., 2010. *Notch signaling in the regulation of stem cell self-renewal and differentiation*, Elsevier Inc.

Lobov, I.B. et al., 2007. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *PNAS*, 104(9), pp.1–6.

Lobov, I.B. et al., 2011. The Dll4/Notch pathway controls postangiogenic blood vessel remodeling and regression by modulating vasoconstriction and blood flow. *Blood*, 117(24), pp.6728–37.

Lu, X. et al., 2004. The netrin receptor UNC5B mediates guidance events controlling morphogenesis of the vascular system. *Nature*, 432(7014), pp.179–186.

Lyden, D. et al., 2001. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nature medicine*, 7(11), pp.1194–1201.

Lymperi, S. et al., 2008. Strontium can increase some osteoblasts without increasing hematopoietic stem cells. *Blood*, 111(3), pp.1173–1181.

Madar, S., Goldstein, I. & Rotter, V., 2013. "Cancer associated fibroblasts" – more than meets the eye. *Trends in Molecular Medicine*, 19(8), pp.447–453.

Maeshima, Y. et al., 2002. Tumstatín, an Endothelial Cell-Specific Inhibitor of Protein Synthesis. *Science*, 295(January), pp.140–144.

Maisonpierre, P.C. et al., 1997. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science*, 277(5322), pp.55–60.

Mandarino, L.J. et al., 1993. Regulation of fibronectin and laminin synthesis by retinal capillary endothelial cells and pericytes in vitro. *Experimental eye research*, 57(5), pp.609–621.

Mantovani, A. et al., 2002. Macrophage polarization: Tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in Immunology*, 23(11), pp.549–555.

Marcus, A.J. et al., 2003. Heterologous cell-cell interactions: thromboregulation, cerebroprotection and cardioprotection by CD39 (NTPDase-1). *Journal of Thrombosis and Haemostasis*, 1(12), pp.2497–2509.

Marcus, A.J. et al., 1991. Inhibition of Platelet Function by an Aspirin-insensitive Endothelial Cell ADPase. *Journal of Clinical Investigation*, 88(November), pp.1690–1696.

Martinez, F.O. & Gordon, S., 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Reports*, 6(March), pp.1–13.

Mavria, G. et al., 2006. ERK-MAPK signaling opposes Rho-kinase to promote endothelial cell survival and sprouting during angiogenesis. *Cancer Cell*, 9(1), pp.33–44.

Mazzone, M. et al., 2009. Heterozygous deficiency of PHD2 restores tumor oxygenation and inhibits metastasis via endothelial normalization. *Cell*, 136(5), pp.839–51.

McCoy, J.M., Wicks, J.R. & Audoly, L.P., 2002. The role of prostaglandin E2 receptors in the pathogenesis of rheumatoid arthritis. *The Journal of clinical investigation*, 110(5), pp.651–8.

McCright, B. et al., 2001. Defects in development of the kidney, heart and eye vasculature in mice homozygous for a hypomorphic Notch2 mutation. *Development (Cambridge, England)*, 128, pp.491–502.

McCright, B., Lozier, J. & Gridley, T., 2002. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. *Development (Cambridge, England)*, 129(4), pp.1075–1082.

Meadows, K.N., Bryant, P. & Pumiglia, K., 2001. Vascular endothelial growth factor induction of the angiogenic phenotype requires Ras activation. *The Journal of Biological Chemistry*, 276(52), pp.49289–98.

Miner, J.H., Cunningham, J. & Sanes, J.R., 1998. Roles for laminin in embryogenesis: Exencephaly, syndactyly,

and placentopathy in mice lacking the laminin alpha-5 chain. *Journal of Cell Biology*, 143(6), pp.1713–1723.

Moncada, S. et al., 1977. Differential formation of prostacyclin (PGX or PGI2) by layers of the arterial wall. An explanation for the anti-thrombotic properties of vascular endothelium. *Thrombosis Research*, 11(3), pp.323–344.

Morrison, S.J. & Scadden, D.T., 2014. The bone marrow niche for haematopoietic stem cells. *Nature*, 505(7483), pp.327–34.

Motta, P.M., 1998. Marcello Malpighi and the foundations of functional microanatomy. *The Anatomical record*, 253(1), pp.10–2.

Mudau, M. et al., 2012. Endothelial dysfunction: the early predictor of atherosclerosis. *Cardiovascular journal of Africa*, 23(4), pp.222–31.

Munro, J.M., Pober, J.S. & Cotran, R.S., 1989. Tumor necrosis factor and interferon-gamma induce distinct patterns of endothelial activation and associated leukocyte accumulation in skin of Papio anubis. *The American journal of pathology*, 135(1), pp.121–33.

Murakami, M., 2012. Signaling required for blood vessel maintenance: molecular basis and pathological manifestations. *International journal of vascular medicine*, 2012, p.293641.

Murakami, M. et al., 2008. The FGF system has a key role in regulating vascular integrity. *Journal of Clinical Investigation*, 118(10), pp.3355–3366.

Nachman, R.L., 2012. Endothelium: from cellophane to orchestral maestro. *The Journal of clinical investigation*, 122(3), pp.796–7.

Nachman, R.L. & Jaffe, E. a., 2004. Endothelial cell culture: beginnings of modern vascular biology. *The Journal of clinical investigation*, 114(8), pp.1037–1040.

Nagasawa, T., 2007. The chemokine CXCL12 and regulation of HSC and B lymphocyte development in the bone marrow niche. *Advances in Experimental Medicine and Biology*, 602, pp.69–75.

Nagy, J.A. et al., 2009. Why are tumour blood vessels abnormal and why is it important to know? *British journal of cancer*, 100(6), pp.865–869.

Nakajima, M. et al., 2003. Abnormal blood vessel development in mice lacking presenilin-1. *Mechanisms of Development*, 120(6), pp.657–667.

Niessen, C.M., 2007. Tight Junctions/Adherens Junctions: Basic Structure and Function. *Journal of Investigative Dermatology*, (January), pp.2525–2532.

Nilsson, I. et al., 2010. VEGF receptor 2/-3 heterodimers detected in situ by proximity ligation on angiogenic sprouts. *The EMBO Journal*, 29(8), pp.1377–1388.

Nishie, a et al., 1999. Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 5(May), pp.1107–1113.

Nitta, T., 2003. Size-selective loosening of the blood-brain barrier in claudin-5-deficient mice. *The Journal of Cell Biology*, 161(3), pp.653–660.

le Noble, F., 2003. Flow regulates arterial-venous differentiation in the chick embryo yolk sac. *Development*, 131(2), pp.361–375.

Nolan, D.J. et al., 2014. Molecular Signatures of Tissue-Specific Microvascular Endothelial Cell Heterogeneity in Organ Maintenance and Regeneration. *Developmental Cell*, 26(2).

Nombela-Arrieta, C. et al., 2013. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nature cell biology*, 15(5), pp.533–43.

O'Reilly, M.S. et al., 1997. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell*, 88(2), pp.277–285.

Oh, H. et al., 1999. Hypoxia and vascular endothelial growth factor selectively up- regulate angiopoietin-2 in bovine microvascular endothelial cells. *Journal of Biological Chemistry*, 274(22), pp.15732–15739.

Ohno, S. et al., 2004. Correlation of histological localization of tumor-associated macrophages with clinicopathological features in endometrial cancer. *Anticancer Research*, 24(5 C), pp.3335–3342.

Oka, C. et al., 1995. Disruption of the mouse RBP-J kappa gene results in early embryonic death. *Development (Cambridge, England)*, 121(10), pp.3291–3301.

Okamoto, T. et al., 2012. Thrombomodulin: a bifunctional modulator of inflammation and coagulation in sepsis. *Critical care research and practice*, 2012(Dic), p.614545.

Omatsu, Y. et al., 2010. The Essential Functions of Adipo-osteogenic Progenitors as the Hematopoietic Stem and Progenitor Cell Niche. *Immunity*, 33(3), pp.387–399.

Paleolog, E.M., 2002. Angiogenesis in rheumatoid arthritis. *Arthritis Res*, 42(7), pp.563–73.

Panés, J. & Granger, D.N., 1998. Leukocyte-endothelial cell interactions: molecular mechanisms and implications in gastrointestinal disease. *Gastroenterology*, 114(5), pp.1066–90.

Park, J.E. et al., 1994. Placenta growth factor: Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *Journal of Biological Chemistry*, 269(41), pp.25646–25654.

Pate, M. et al., 2010. *Endothelial Cell Biology: Role in The Inflammatory Response* 1st ed., Elsevier Inc.

Patel, N.S., 2005. Up-regulation of Delta-like 4 Ligand in Human Tumor Vasculature and the Role of Basal Expression in Endothelial Cell Function. *Cancer Research*, 65(19), pp.8690–8697.

Peng, G. & Liu, Y., 2015. Hypoxia-inducible factors in cancer stem cells and inflammation. *Trends in Pharmacological Sciences*, 36(6), pp.374–383.

Phng, L.K. & Gerhardt, H., 2009. Angiogenesis: A Team Effort Coordinated by Notch. *Developmental Cell*, 16.

Pober, J.S., Min, W. & Bradley, J.R., 2009. Mechanisms of Endothelial Dysfunction, Injury, and Death. *Annual Review of Pathology: Mechanisms of Disease*, 4(1), pp.71–95.

Pober, J.S. & Sessa, W.C., 2007. Evolving functions of endothelial cells in inflammation. *Nature Reviews Immunology*, 7(10), pp.803–815.

Pöschl, E. et al., 2004. Collagen IV is essential for basement membrane stability but dispensable for initiation of its assembly during early development. *Development (Cambridge, England)*, 131(7), pp.1619–1628.

Poulos, M.G. et al., 2013. Endothelial Jagged-1 is necessary for homeostatic and regenerative hematopoiesis. *Cell Reports*, 4(5), pp.1022–1034.

Praagh, R. Van & Praagh, S. Van, 1983. Aristotle's "triventricular" heart and the relevant early history of the cardiovascular system. *Chest*, 84(4), pp.462–468.

Presta, M. et al., 2005. Fibroblast growth factor/fibroblast growth factor receptor system in angiogenesis. *Cytokine & Growth Factor Reviews*, 16(2), pp.159–178.

Pugh, C.W. & Ratcliffe, P.J., 2003. Regulation of angiogenesis by hypoxia: role of the HIF system. *Nature medicine*, 9(6), pp.677–84.

Rafii, D.C. et al., 2008. Regulation of vasculogenesis by platelet-mediated recruitment of bone marrow-derived cells. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 28(2), pp.217–222.

Rajendran, P. et al., 2013. The Vascular Endothelium and Human Diseases. *International Journal of Biological Sciences*, 9(10), pp.1057–1069.

Ramseyer, V., Hong, N. & Garvin, J.L., 2012. Tumor Necrosis Factor Alpha decreases NOS3 expression primarily via Rho/Rho Kinase in the thick ascending limb. *Hypertension*, 18(9), pp.1199–1216.

Red-Horse, K. et al., 2007. Endothelium-Microenvironment Interactions in the Developing Embryo and in the Adult. *Developmental Cell*, 12(2), pp.181–194.

Reedijk, M. et al., 2005. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Research*, 65(18), pp.8530–8537.

Ribatti, D., 2008. Judah Folkman, a pioneer in the study of angiogenesis. *Angiogenesis*, 11(1), pp.3–10.

Ribatti, D. et al., 2001. Postnatal vasculogenesis. *Mechanisms of Development*, 100(2), pp.157–163.

Ribatti, D. & Crivellato, E., 2012. "Sprouting angiogenesis", a reappraisal. *Developmental Biology*, 372(2), pp.157–165.

Ridgway, J. et al., 2006. Inhibition of Dll4 signalling inhibits tumour growth by deregulating angiogenesis. *Nature*, 444(December), pp.1–5.

Risau, W. & Flamme, I., 1995. Vasculogenesis. *Annual review of cell and developmental biology*, 11, pp.73–91.

Roy, H., Bhardwaj, S. & Ylä-Herttuala, S., 2006. Biology of vascular endothelial growth factors. *FEBS Letters*, 580(12), pp.2879–2887.

Saharinen, P. et al., 2005. Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2. *Journal of Cell Biology*, 169(2), pp.239–243.

Saitou, M. et al., 2000. Complex phenotype of mice lacking occludin, a component of tight junction strands. *Molecular biology of the cell*, 11(12), pp.4131–4142.

Sakata, Y. et al., 2002. Ventricular septal defect and cardiomyopathy in mice lacking the transcription factor CHF1/Hey2. *Proceedings of the National Academy of Sciences of the United States of America*, 99(25), pp.16197–16202.

Sandoo, A. et al., 2010. The endothelium and its role in regulating vascular tone. *The open cardiovascular medicine journal*, 4, pp.302–312.

Santagata, S. et al., 2004. JAGGED1 Expression Is Associated with Prostate Cancer Metastasis and Recurrence. *Cancer Research*, 64(617), pp.6854–6857.

Sato, T.N. et al., 1995. Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature*, 376, pp.70–74.

Schofield, R., 1978. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood cells*, 4(1–2), pp.7–25.

Seegar, T.C.M. et al., 2010. Tie1-Tie2 interactions mediate functional differences between angiopoietin ligands. *Molecular cell*, 37(5), pp.643–55.

Seghezzi, G. et al., 1998. Fibroblast growth factor-2 (FGF-2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: An autocrine mechanism contributing to angiogenesis. *Journal of Cell Biology*, 141(7), pp.1659–1673.

Shalaby, F. et al., 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*, 376(6535), pp.62–6.

Shawber, C.J. et al., 2007. Notch alters VEGF responsiveness in human and murine endothelial cells by direct regulation of VEGFR-3 expression. *Journal of Clinical Investigation*, 117(11), pp.3369–3382.

Shi, Q. et al., 1998. Evidence for circulating bone marrow-derived endothelial cells. *Blood*, 92(2), pp.362–367.

Shibuya, M., 2001. Structure and dual function of vascular endothelial growth factor receptor-1 (Flt-1). *The international journal of biochemistry & cell biology*, 33, pp.409–420.

Shibuya, M. & Claesson-Welsh, L., 2006. Signal transduction by VEGF receptors in regulation of angiogenesis and lymphangiogenesis. *Experimental Cell Research*, 312(5), pp.549–560.

Shoja, M.M. et al., 2008. The Aristotelian account of "heart and veins." *International Journal of Cardiology*, 125(3), pp.304–310.

Sica, A. & Mantovani, A., 2012. Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation*, 122(3), pp.787–795.

Siekmann, A.F. & Lawson, N.D., 2007. Notch signalling limits angiogenic cell behaviour in developing zebrafish arteries. *Nature*, 445(7129), pp.781–784.

Sörensen, I., Adams, R.H. & Gossler, A., 2009. DLL1-mediated Notch activation regulates endothelial identity in mouse fetal arteries. *Blood*, 113(22), pp.5680–5688.

Soriano, P., 1994. Abnormal kidney development and hematological disorders in PDGF beta-receptor mutant mice. *Genes & development*, 8(16), pp.1888–1896.

Sprague, A.H. & Khalil, R.A., 2010. Inflammatory Cytokines in Vascular Dysfunction and Vascular Disease. *Biochem Pharmacol.*, 78(6), pp.539–552.

Stapleton, S., 2009. The History of Tumour Angiogenesis as a Therapeutic Target. *University of Toronto Medical Journal*, 87(1), pp.45–49.

Steyers, C. & Miller, F., 2014. Endothelial Dysfunction in Chronic Inflammatory Diseases. *International Journal of Molecular Sciences*, 15(7), pp.11324–11349.

Stratman, A.N., Davis, M.J. & Davis, G.E., 2011. VEGF and FGF prime vascular tube morphogenesis and sprouting directed by hematopoietic stem cell cytokines. *Blood*, 117(14), pp.3709–3719.

Strilić, B. et al., 2009. The Molecular Basis of Vascular Lumen Formation in the Developing Mouse Aorta. *Developmental Cell*, 17(4), pp.505–515.

Suchting, S. et al., 2007. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proceedings of the National Academy of Sciences of the United States of America*, 104(9), pp.3225–3230.

Sugiyama, T. et al., 2006. Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. *Immunity*, 25, pp.977–988.

Sugiyama, T. & Nagasawa, T., 2012. Bone marrow niches for hematopoietic stem cells and immune cells. *Inflammation & allergy drug targets*, 11(3), pp.201–6.

Suri, C. et al., 1996. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell*, 87(7), pp.1171–1180.

Tabas, I. & Glass, C.K., 2013. Anti-Inflammatory Therapy in Chronic Disease: Challenges and Opportunities. *Science*, 339(January), pp.166–172.

Tammela, T. et al., 2008. Blocking VEGFR-3 suppresses angiogenic sprouting and vascular network formation. *Nature*, 454(7204), pp.656–60.

Tammela, T. et al., 2011. VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling. *Nature Cell Biology*, 13(10), pp.1202–1213.

Tepper, O.M. et al., 2005. Adult vasculogenesis occurs through in situ recruitment, proliferation, and tubulization of circulating bone marrow-derived cells. *Blood*, 105(3), pp.1068–1077.

Thurston, G. et al., 2000. Angiopoietin-1 protects the adult vasculature against plasma leakage. *Nature medicine*, 6(4), pp.460–463.

Tille, J.C. et al., 2001. Vascular endothelial growth factor (VEGF) receptor-2 antagonists inhibit VEGF- and basic fibroblast growth factor-induced angiogenesis in vivo and in vitro. *The Journal of pharmacology and*

experimental therapeutics, 299(3), pp.1073–1085.

Tokoyoda, K. et al., 2004. Cellular niches controlling B lymphocyte behavior within bone marrow during development. *Immunity*, 20(6), pp.707–718.

Tolins, J.P. et al., 1991. Calcium Channel Blockade Inhibits Platelet Activating Factor Production by Human Umbilical Vein Endothelial Cells 1. , 26(12), pp.1218–1222.

Toro, R. Del et al., 2011. Identification and functional analysis of endothelial tip cell − enriched genes. *Blood*, 116(19), pp.4025–4033.

Torres-Vázquez, J., Kamei, M. & Weinstein, B.M., 2003. Molecular distinction between arteries and veins. *Cell and Tissue Research*, 314(1), pp.43–59.

Totoson, P. et al., 2014. Mechanisms of endothelial dysfunction in rheumatoid arthritis: lessons from animal studies. *Arthritis research & therapy*, 16(1), p.202.

Trindade, A. et al., 2008. Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos. *Blood*, 112(5), pp.1720–9.

Tzeng, Y.S. et al., 2011. Loss of Cxcl12/Sdf-1 in adult mice decreases the quiescent state of hematopoietic stem/progenitor cells and alters the pattern of hematopoietic regeneration after myelosuppression. *Blood*, 117(2), pp.429–439.

Uyttendaele, H. et al., 2001. Vascular patterning defects associated with expression of activated Notch4 in embryonic endothelium. *Proceedings of the National Academy of Sciences of the United States of America*, 98(10), pp.5643–5648.

Visnjic, D. et al., 2004. Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood*, 103(9), pp.3258–3264.

Wacker, A. & Gerhardt, H., 2011. Endothelial development taking shape. *Current Opinion in Cell Biology*, (Cv).

Wallez, Y. & Huber, P., 2008. Endothelial adherens and tight junctions in vascular homeostasis, inflammation and angiogenesis. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1778(3), pp.794–809.

Waltenberger, J. et al., 1994. Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *Journal of Biological Chemistry*, 269(43), pp.26988–26995.

Wang, D. & DuBois, R.N., 2004. Cyclooxygenase 2-derived prostaglandin E2 regulates the angiogenic switch. *Proceedings of the National Academy of Sciences*, 101(2), pp.415–416.

Wang, Q. et al., 2012. Notch2 and Notch3 function together to regulate vascular smooth muscle development. *PloS one*, 7(5), p.e37365.

Wang, Y.C. et al., 2010. Notch signaling determines the M1 versus M2 polarization of macrophages in antitumor immune responses. *Cancer Research*, 70(12), pp.4840–4849.

Warner, K. a et al., 2008. Endothelial cells enhance tumor cell invasion through a crosstalk mediated by CXC chemokine signaling. *Neoplasia (New York, N.Y.)*, 10(2), pp.131–9.

Weber, C., Fraemohs, L. & Dejana, E., 2007. The role of junctional adhesion molecules in vascular inflammation. *Nature Reviews Immunology*, 7(6), pp.467–477.

Weinstein, B.M., 1999. What guides early embryonic blood vessel formation? *Developmental dynamics*, 215(1), pp.2–11.

Weis, S.M. & Cheresh, D.A., 2011. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med*, 17(11), pp.1359–1370.

Whatley, R.E. et al., 1990. Synthesis of Platelet-activating Factor by Endothelial Cells. *Journal of Biological Chemistry*, 265, No 26(26), pp.15550–15559.

Wheelock, M.J. & Johnson, K.R., 2003. Cadherin-mediated cellular signaling. *Current Opinion in Cell Biology*, 15(5), pp.509–514.

Williams, C.K. et al., 2006. Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. *Blood*, 107(3), pp.931–939.

Winkler, E.A., Bell, R.D. & Zlokovic, B. V, 2011. Central nervous system pericytes in health and disease. *Nature Neurosciences*, 14(11), pp.1398–1405.

Winkler, E.A., Bell, R.D. & Zlokovic, B. V, 2010. Pericyte-specific expression of PDGF beta receptor in mouse models with normal and deficient PDGF beta receptor signaling. *Molecular neurodegeneration*, 5, p.32.

Woods, J.M. et al., 2003. The role of COX-2 in angiogenesis and rheumatoid arthritis. *Experimental and Molecular Pathology*, 74(3), pp.282–290.

Xue, Y. et al., 1999. Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Human molecular genetics*, 8(5), pp.723–730.

Yana, I. et al., 2007. Crosstalk between neovessels and mural cells directs the site-specific expression of MT1-MMP to endothelial tip cells. *Journal of Cell Science*, 120(9), pp.1607–1614.

Yang, L.-T., 2004. Fringe Glycosyltransferases Differentially Modulate Notch1 Proteolysis Induced by Delta1 and Jagged1. *Molecular Biology of the Cell*, 16(2), pp.927–942.

Yao, L. et al., 2005. Bone marrow dysfunction in mice lacking the cytokine receptor gp130 in endothelial cells. *Blood*, 106(13), pp.4093–4101.

Yau, J.W., Teoh, H. & Verma, S., 2015. Endothelial cell control of thrombosis. *BMC Cardiovascular Disorders*, 15(1), p.130.

Yoo, S.-A. et al., 2009. Role of placenta growth factor and its receptor flt-1 in rheumatoid inflammation: a link between angiogenesis and inflammation. *Arthritis and Rheumatism*, 60(2), pp.345–54.

Yoo, S.Y. & Kwon, S.M., 2013. Angiogenesis and its therapeutic opportunities. *Mediators of inflammation*, 2013(1), p.127170.

You, L.-R. et al., 2005. Suppression of Notch signalling by the COUP-TFII transcription factor regulates vein identity. *Nature*, 435, pp.0–6.

Yusuf-Makagiansar, H. et al., 2002. Inhibition of LFA-1/ICAM-1 and VLA-4/VCAM-1 as a therapeutic approach to inflammation and autoimmune diseases. *Medicinal Research Reviews*, 22(2), pp.146–167.

Zarkada, G. et al., 2015. VEGFR3 does not sustain retinal angiogenesis without VEGFR2. *Proceedings of the National Academy of Sciences*, 112(3), p.201423278.

Zeisberg, E.M. et al., 2007. Discovery of Endothelial to Mesenchymal Transition as a Source for Carcinoma-Associated Fibroblasts. *Cancer Research*, 67(21), pp.10123–10128.

Zhang, L. et al., 2010. VEGFR-3 ligand-binding and kinase activity are required for lymphangiogenesis but not for angiogenesis. *Cell research*, 20(12), pp.1319–1331.

Zhang, R. et al., 2003. Etk/Bmx transactivates vascular endothelial growth factor 2 and recruits phosphatidylinositol 3-kinase to mediate the tumor necrosis factor-induced angiogenic pathway. *J Biol Chem*, 278(51), pp.51267–51276.

Zhou, X. et al., 2010. Multiple functions of Osterix are required for bone growth and homeostasis in postnatal mice. *Proceedings of the National Academy of Sciences of the United States of America*, 107(29), pp.12919–12924.

Zhu, J. et al., 2007. Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. *Blood*, 109(9), pp.3706–12.

2.

ENDOTHELIAL JAGGED 1 ENHANCES MACROPHAGE RECRUITMENT AND ALTERNATIVE ACTIVATION IN A SETTING OF PROSTATE ADENOCARCINOMA

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CONTENTS

2.1. ABSTRACT

Macrophages are a major cellular component of the tumor microenvironment, which is capable of modifying them in a "polarized" manner: "classically" activated (M1) macrophages promote pro-inflammatory and anti-tumoral responses, whereas "alternatively" activated (M2) macrophages exert pro-tumoral and pro-angiogenic functions. The signals that polarize macrophages in the tumor microenvironment are not completely undisclosed and were the subject of this Chapter. Endothelial cells and macrophages engage in specific interactions that not only modulate the tumor angiogenic properties but are also thought to affect macrophage polarization. We used two conditional mouse models with endothelial-specific Jagged 1 (eJag1) loss- or gain-of-function (eJag1^{KO} and eJag1^{OE}) to address the role of eJag1 in tumor-associated macrophage recruitment and polarization. Our data shows that eJag1 positively regulates macrophage recruitment and M2 polarization in the transgenic adenocarcinoma mouse prostate (TRAMP) mouse model. Both *in vivo* Jag1 conditional knockout and *in vitro* Jag1 inhibition in HUVECs decreased macrophage polarization into a pro-tumoral activation state. Endothelial Jag1 modulation affected the angiocrine expression of adhesion molecules and chemoattractants, upregulating *Angpt2* in both mouse models and decreasing *Cxcl12* and *Vcam1* only in eJag1^{OE} mice. Expression of other endothelial Notch ligands was affected in eJag1^{OE} mice, but not in eJag1^{KO} mice, which had higher levels of *Dll4* and *Jag2* and *Dll1* downregulation. Similarly, macrophage gene expression was also affected by endothelial Jag1, whose levels positively correlated with that of *Il6* and *Tnfa* transcription, and negatively correlated with *Notch2* expression levels, particularly in M2 macrophages. Furthermore, M2 macrophages showed a general increase in *Notch1* and *Cxcr4* expression, irrespective of eJag1 modulation. Together, our data suggests that endothelial Jag1 induces the recruitment of tumor-associated macrophages and their polarization into a pro-tumoral state through the modulation of angiocrine genes which, in turn, induces the modification of the macrophage gene expression patterns.

2.2. INTRODUCTION

The contemporary view of cancer envisions tumors as complex systems. Solid tumors are increasingly accepted to comprise not only malignant cells, but also many other non-malignant cell types that interact with each other and with the tumor cells, creating a unique microenvironment that can modify tumor cell properties. Endothelial cells and infiltrating cells of the immune system are two of the stromal constituents of solid tumors.

During tumor progression, circulating monocytes and macrophages are actively recruited into tumors, shifting their phenotypes in response to the pleiotropy of microenvironmental signals released by the tumor and stromal cells. Depending on their stimuli, macrophages may undergo classical (M1) or alternative (M2) activation (Gordon & Martinez 2010). M1 macrophages are induced in response to lipopolysaccharide (LPS), interferon-γ (IFN-γ), and Toll-like receptor (TLR) agonists, and produce pro-inflammatory cytokines such as IL6, IL12, and tumor necrosis factor-α (TNF-α), serving as a critical cellular component both in the inflammatory response and in antitumor immunity (Sica & Mantovani 2012). Conversely, M2 macrophages are induced by apoptotic cells, IL4, IL10, IL13 and tumor growth factor-β (TGFβ), among others (Murray et al. 2014), and are involved in the resolution of the inflammatory response, wound healing, and pro-tumorigenic properties (Rőszer 2015). M2 macrophages are characterized by the expression of Arginase 1 and mannose receptor (CD206), and secrete a variety of pro-angiogenic factors, such as VEGF, FGF and matrix metalloproteinases (Chanmee et al. 2014). Tumor-associated macrophages (TAMs) closely resemble M2-polarized macrophages and are crucial modulators of the tumor microenvironment, generally promoting tumor development and progression (Chanmee et al. 2014). Consistently, extensive infiltration of TAMs in many solid tumors has been correlated with poor prognosis and increased tumor angiogenesis (Leek et al. 1996; Nishie et al. 1999; Koide et al. 2004; Hanada et al. 2000; He et al. 2012; Espinosa et al. 2011; Ohno et al. 2004).

The interactions between endothelial cells and hematopoietic cells have been shown to be critical for the trafficking and homing of hematopoietic cells (He et al. 2014), as well as for recruitment and activation of inflammatory cells to specific tissue sites (Cook-Mills & Deem 2005). Recently, endothelial cells were also shown to support the differentiation of M2 macrophages directly from hematopoietic progenitor cells (He et al. 2012), suggesting

that the macrophage-endothelial cell interactions were bidirectional, inducing both pro-angiogenic macrophage development and differentiation and macrophage-dependent angiogenesis (He et al. 2014; Baer et al. 2013).

The Notch signaling pathway is highly conserved and is involved in several cell fate specifications throughout development (Lai 2004). Several studies have suggested that Notch signaling is implicated in macrophage recruitment and activity in vessel anastomosis (Outtz et al. 2011; Tattersall et al. 2016; Outtz et al. 2010; Fung et al. 2007). Furthermore, Notch signaling seems to be of critical importance in the determination of M1 versus M2 polarization in tumors. In fact, Notch activation was demonstrated to induce M1 polarization, even in the presence of an M2 inducer, and the opposite was found upon blockade of Notch signaling, which resulted in M2 polarization, even in the presence of M1 inducers (Wang et al. 2010). However, little is known about the involvement of this pathway in macrophage-endothelial cells bidirectional interactions.

We sought to characterize the macrophage recruitment and polarization, as well as the modulation of macrophage and endothelial cells gene expression patterns in endothelial Jag1 mutant mice that develop spontaneous prostate tumors (TRAMP) (Gingrich & Greenberg 1996; Gingrich et al. 1996). Our results show that, consistent with the observations that endothelial Jag1 increased tumor growth and angiogenesis (A.-R. Pedrosa et al. 2015), it also enhanced macrophage recruitment into the tumors, possibly through endothelial-derived *Angpt2* expression, and promoted polarization towards a pro-tumoral (M2) state. Moreover, the pro-angiogenic effect of the polarized macrophages was likely induced by their IL6 and TNFα increased production.

2.3. METHODS

2.3.1. Animal experiments

All experimental animal procedures in this study were approved by the Ethics and Animal Welfare Committee of the Faculty of Veterinary Medicine of Lisbon and executed in strict compliance to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA).

The generation of both TRAMP*Jag1$^{lox/lox}$*VE-Cadherin-Cre-ERT2 and TRAMP*Tet-O-Jag1*Tie2-rtTA has been previously described (A.-R. Pedrosa et al. 2015). Briefly, to obtain the loss-of-function mice Jag1$^{lox/+}$ mice (in which the coding region for the DSL (Delta-Serrate-Lag2) region of Jag1 is flanked by loxP sites) (Kiernan et al. 2006) were crossed with VE-Cadherin-CRE-ERT2 mice (Monvoisin et al. 2006), generating a conditional "knockout" mice. The resulting progeny was then crossed with a mouse model of prostate adenocarcinoma (TRAMP), and TRAMP*Jag1$^{lox/lox}$*VE-Cadherin-Cre-ERT2 (eJag1^{KO}) were obtained. Endothelial-specific Jag1 depletion was achieved upon intraperitoneal (IP) treatment with tamoxifen (50 mg/kg/day in castor oil, Sigma) for 5 consecutive days, in mice with 8 weeks of age. Control mice (Control KO) had the same *Jag1* loss-of-function genotype but were not induced with tamoxifen.

To generate the gain-of-function mutants, heterozygous Tet-O-Jag1 mice were crossed with a line of heterozygous Tie2-rtTA mutant mice. The resulting progeny was crossed with TRAMP mice, and TRAMP*Tet-O-Jag1*Tie2-rtTA (eJag1^{OE}) mice were obtained. To induce Jag1 overexpression under the control of the Tie2 promotor, mice were induced through administration of doxycyclin (4mg/mL, Sigma) in drinking water from week 8, and throughout the entire experiments. Control mice (Control OE) had the same gain-of-function genotype and developed prostate tumors but were not induced with doxycycline. In all TRAMP models, eJag1^{KO}, eJag1^{OE} and respective controls, prostates were dissected at 18 (early stage) or 24 (late stage) weeks of age and processed for immunostaining.

2.3.2. Flow cytometry and cell sorting

For flow cytometry analysis and sorting of endothelial cells and macrophages, prostates were collected, finely dissected into small pieces (2-4 mm) and digested with 1mL solution of 1% collagenase (Sigma) and 2,4U/mL dispase (Gibco, Life Technologies). The digestion was performed at 37ºC for 2h30min, with agitation, and DNAse I (Sigma) was added in the last half hour of digestion to eliminate DNA residues. Cells were washed with PBS and filtered with cell strainers (40 µm pore size) and were then subjected to immunostaining for anti-CD31 (MEC13.3) Fluorescein Isothiocyanate (FITC) (BD Biosciences), anti-F4/80 (BM8) Phycoerythrin (PE), anti-Ter119 (TER-119) PE-Cyanine 7 (PE/Cy7), anti-CD45 (30-F11) PE/Cy7 (all from eBiosciences), anti-CD11b (M1/70)

Allophycocyanin (APC), anti-MHCII (M5/114.15.2) APC/Cy7 and CD206 (C068C2) FITC (all from BioLegend). Cell sorting of endothelial cells ($CD31^+CD45^-Ter119^-$), M1 macrophages ($CD11b^+/F4/80^+/MHCII^+/CD206^-$) and M2 macrophages ($CD11b^+/F4/80^+/MHCII^+/CD206^+$) was performed with an Aria Cell Sorter equipped with FACS Diva 6.2 Software (BD Biosciences). Dead cells and debris were excluded by FSC, SSC and 7AAD (7-Amino-Actinomycin D) cell viability solution (BD Biosciences) staining profiles and sorted cells were collected into TRIzol Reagent (Invitrogen). $CD45^+$ were also sorted and stained with anti-CD19 (1D3) APC (BD Biosciences), anti-CD3e (145-2C11) PE (eBiosciences) and anti-CD8 (53-6.7) PerCP for flow cytometric analysis of prostate lymphocytic components.

To sort cells for the macrophage polarization assay, BM cells were flushed with PBS 2mM EDTA, treated with Red Blood Cell Lysis Buffer (Biolegend) for 15 minutes in the dark and $CD11b^+$ cells were purified using the $CD11b^+$ Microbead Kit (Miltenyi Biotec) according to the manufacturer's protocol. Analysis of $CD11b^+$ cells to detect M1-($CD11b^+/F4/80^+/MHCII^+/CD206^-$) and M2-like ($CD11b^+/F4/80^+/MHCII^+/CD206^+$) macrophages was then carried out using an LSR Fortessa flow cytometer equipped with FACS Diva 6.2 Software (BD Biosciences). Dead cells and debris were excluded by FSC, SSC and 7AAD (7-Amino-Actinomycin D) cell viability solution (BD Biosciences) staining profiles. Data were analyzed with a FlowJo 9.8.2 Software.

2.3.3. Cell culture and macrophage polarization assay

Human umbilical cord vein endothelial cells (HUVECs) (Clonetics, Lonza) were cultured in 24-well plates (Corning) at a density of $1x10^5$ cells/mL in EBM-2 supplemented with EGM-2 Single-Quots, 2mg/mL BBE (Lonza) and 1% heat-inactivated fetal bovine serum (FBS) (Gibco). To inhibit Delta-like 4 (Dll4)- and Jag1-mediated Notch signaling, 24h after plating, cells were treated with 20µg/mL of neutralizing anti-human Dll4 antibody (MHD4-46) (Sunamura & Yagita 2008) and/or anti-human Jag1 antibody (MHJ1-152) (Sekine et al. 2012) (kindly provided by Dr. Hideo Yagita) for 24 hours. Equal quantity of PBS was added to the controls. After 24 hours of culture with the neutralizing antibodies, cells were washed with PBS and $1x10^5$ isolated $CD11b^+$ cells were placed on top of the pre-plated HUVECs in RPMI 1%FBS. To maintain the Notch signaling inhibition, anti-human Dll4 and Jag1 antibodies were added to the co-cultures. As control, $1x10^5$ $CD11b^+$ cells were plated

alone and treated with the same concentration of the Notch ligands neutralizing antibodies. $CD11b^+$ cells were also cultured with conditioned media from 24 hours-treated HUVECs. Four days after plating, cells were harvested with 0,25% Trypsin-EDTA (Gibco), washed in PBS and stained for flow cytometric analysis.

2.3.4. RNA isolation and quantitative PCR

RNA was extracted according to Invitrogen's instructions for TRIzol Reagent. Reverse transcription was performed with SuperScript II (Invitrogen), using Random Primers (Sigma) and according to the manufacturer's protocol. Quantitative PCR was performed with Power SYBR Green PCR Master Mix (Roche) on a ViiA™ 7 Real-Time PCR System (Life Technologies). The sequences of the oligonucleotides used are included in **Table 2.I**. A primer concentration of 180nM was found to be optimal in all cases. Amplification of beta-2-microglobulin (*B2m*) was used for sample normalization.

Table 2.I. Primers list.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
m***B2m***	TCACGCCACCCACCGGAGAA	TGTGAGGCGGGTGGAACTGTG
m***Angpt2***	CAGCAGCACAAACTCGGAAAC	TCGAGTCTTGTCGTCTGGTTTAG
m***Cxcl12***	GCCAACGTCAAGCATCTGAAAA	TCTTCAGCCGTGCAACAATC
m***Cxcr4***	GGGACATCAGTCAGGGGGAT	CTATCGGGGTAAAGGCGGTC
m***Dll1***	CGATTCCCCTTCGGCTTCAC	GGGTTTTCTGTTGCGAGGTC
m***Dll4***	TTTGTGACCAAGATCTCAACTACTGTAC	CTTTGGCCCACTGTTGGAA
m***Il6***	CTGGGAAATCGTGGAAATGAGA	GCAAGTGCATCATCGTTGTTCAT
m***Jagged2***	TCATTCCCTTTCAGTTCGCC	CCTCATCTGGAGTGGTGTCATT
m***Notch1***	CGGTGAACAATGTGGATGCT	ACTTTGGCAGTCTCATAGCT
m***Notch2***	ACGCAGTCCAGTTGCCCTCCA	GGGCAAACGGGGCATCTCTGG
m***Tnfa***	GGTCCCCAAAGGGATGAGAA	TGAGGGTCTGGGCCATAGAA
m***Vcam1***	GCCTCAACGGTACTTTGGATA	TGGAGTCACCGATTTGAGCAAT
m***Vegfa***	GTACCTCCACCATGCCAAGT	TCTGCTCTCCTTCTGTCGTG

2.3.5. Statistical analysis

Data processing was carried out using Graph Pad Prism 6 Software and statistical analysis was performed using unpaired two-tailed Student's t test. Results are expressed as mean ± standard deviation. P values of <0.05 were considered statistically significant.

2.4. RESULTS

2.4.1. Modulation of endothelial Jag1 affects the recruitment of immune cells to the tumor site

Endothelial Jag1 (eJag1) contribution to prostate cancer progression has been recently addressed by Pedrosa and colleagues, who demonstrated that eJag1 is required for prostate cancer development by inducing both tumor angiogenesis and vessel maturation (A.-R. Pedrosa et al. 2015). However, their study failed to unravel whether eJag1 modulation could change the type of immune cells that are recruited to the tumor site and thereby promote tumor growth through distinct mechanisms. Understanding the immune system's role in modulation of solid tumors has increased significantly in recent years, and it is now known that various immune cells are found within the tumor site, including T-cells, B-cells, macrophages, and neutrophils, among others, which may have either tumor-suppressor or tumor-promoting activities.

To explore whether endothelial Jag1 was modulating the amount of tumor infiltrating lymphocytes or macrophages, we used the previously described mutant mice resulting from the crosses of endothelial Jag1 mutants with a prostate adenocarcinoma mouse model (TRAMP) (A.-R. Pedrosa et al. 2015). TRAMP mice develop prostatic lesions from 8 weeks of age (Kaplan-Lefko et al. 2003), and thus the generated mice, eJag1^{OE} and eJag1^{KO} and the respective controls, were sacrificed at 18 and 24 weeks of age, representing an early and a late stage of prostate cancer development.

Preliminary data from the analysis of the prostate B and T lymphoid composition within previously isolated CD45^{+} leukocytes suggest that knocking out Jag1 in endothelial cells decreases both total T cells (CD3^{+}) and cytotoxic T cells (CD3^{+}/CD8^{+}) at an early stage of prostate cancer development. However, at a later stage (24w) these mice showed a significant increase in CD3^{+} T cells (15.13% ± 1.20 in eJag1^{KO}), but not in CD8^{+} T cells, compared to Control KO mice (10.93% ± 0.96) (**Figure 2.1B and C**). This suggests that at the later stage eJag1^{KO} mice have an increase in CD4^{+} T cells, most likely in T helper cells which similarly to CD8^{+} T cells have been shown to exert potent anti-tumor activity (Kim & Cantor 2014; Zanetti 2015) and may have a role in the decreased tumor size observed in these mice compared to their controls.

eJag1^{OE} mice, on the other hand, did not show any differences at the earlier stage, but seem to have an increase in both CD19^{+} B and CD3^{+} T cells in the later stage (**Figure 2.1A and B**), without changes in the percentage of cytotoxic T cells (**Figure 2.1C)**. In these mice, the increase in total T cells might be a consequence of increased regulatory T cells (Tregs), consistent with previous findings reporting that Jag1 signaling through Notch3 is essential for Treg induction and expansion (Gopisetty et al. 2013). Tregs, a subtype of CD4^{+} T cells, are known to accumulate in the tumor environment and to suppress tumor-specific T-cell responses (Kim & Cantor 2014; Yu & Fu 2006), and have been shown to be associated with poor prognosis in prostate cancer patients (Davidsson et al. 2013), suggesting they may be contributing for the enhanced tumor growth found in eJag1^{OE} mice. Although B cell infiltration promotes a favorable prognosis in melanoma, breast, lung, and ovarian cancers

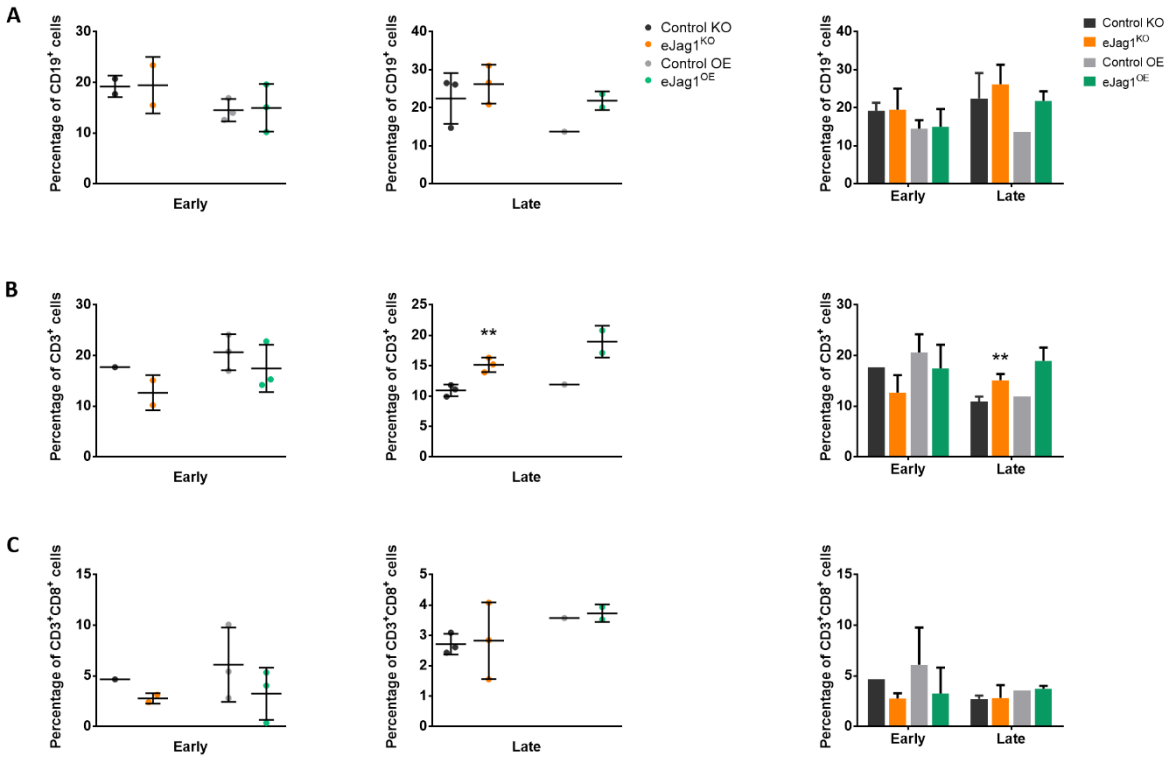

Figure 2.1. eJag1 modulates the percentage of lymphoid cells that are found in the tumor site

Flow cytometric analysis of the prostate tumors in both early and late stages of tumor development suggests that **(A)** CD19^{+} B cells are increased in eJag1^{OE} in the later stage. **(B)** CD3^{+} T cells seem to decrease in the early stage in eJag1^{KO}, but significantly increase in the late stage. The percentage of tumor associated T cells at 24w (late stage) in eJag1^{OE} mice also seem to be increased. **(C)** Analysis of CD3^{+}/CD8^{+} cytotoxic T cells suggests that knocking out Jag1 in endothelial cells modulate the amount of these cells in the tumor at an early stage, but not at a later stage. No differences in cytotoxic T cells were found in eJag1^{OE} mice and their controls (Control OE). Data are represented as mean ± SD (** p<0.01).

(Page et al. 2014; Nielsen et al. 2012; Schmidt et al. 2008; Erdag et al. 2012), a number of studies have demonstrated that B cells can have a pro-tumoral activity through the expression of soluble mediators, such as chemokines and immunoglobulins (Gunderson & Coussens 2013; Spaner & Bahlo 2011), and their increase in eJag1^{OE} mice may contribute to prostate tumor growth and progression.

Similar to Tregs, increased tumor-associated macrophages (TAMs) are usually associated with advanced tumor progression and poor prognosis (Pollard 2004; Bingle et al. 2002). Therefore, we analyzed the macrophage composition of the prostate tumors in the Jag1 mutant mice to understand whether the increased tumor sizes found upon Jag1-endothelial overexpression were linked to increased TAM density within the tumors. The macrophage composition was analyzed using CD11b and F4/80 markers to discriminate TAMs from the remaining cells (**Figure 2.2A**) (Bain et al. 2014; Roth et al. 2012; Jinushi et al. 2011).

Interestingly, our preliminary data suggest that the percentage of TAMs within the isolated prostate tumors was increased in eJag1^{KO} mice in the early stage, but these differences were absent in the late stage. In eJag1^{OE} mice, however, TAMs percentage was similar in the mutant and control mice in the early (18w) stage, but in the late (24w) stage, eJag1^{OE} mice revealed an increased TAM percentage (12.70% ± 0.28) when compared to the Control OE mice (8.15% ± 0.62). (**Figure 2.2A, C and C'**).

Next, we addressed whether the tumors had functionally distinct TAM subsets, by determining the percentage of M1 (MHC II^{+}/CD206^{-}) and M2 (MHC II^{+}/CD206^{+}) macrophages. The data obtained thus far suggest that M1 macrophages are increased in eJag1^{KO} mice both in the early (18w) and late (24w) stages of tumor development, compared to the Control KO mice. Furthermore, these mice showed lower levels of M2 macrophages. The exact opposite was found in eJag1^{OE} mice, which had lower levels of M1 macrophages and increased M2 macrophages within the prostate tumor in both stages of the tumor development.

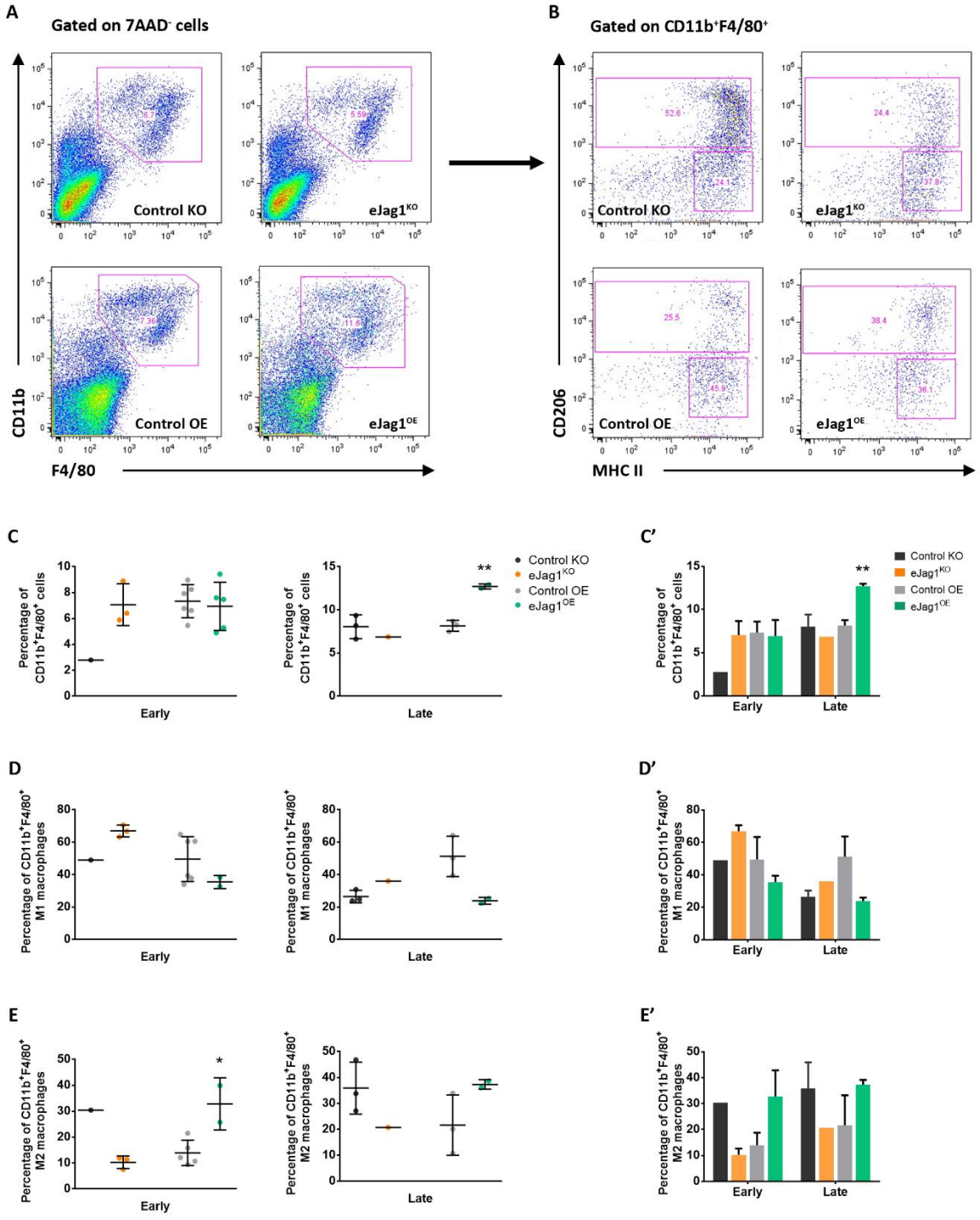

Figure 2.2. Endothelial Jag1 affects macrophage polarization towards an M2-like phenotype

(A-B) Representative plots of the flow cytometric analysis of isolated prostate tumors from eJag1^{KO}, eJag1^{OE} and respective controls. Analysis of the percentages of **(C and C')** CD11b^{+}/F4/80^{+} tumor associated macrophages (TAMs) suggests that eJag1^{KO} have higher numbers of macrophages in the early state, but not in the late stage. eJag1^{OE}, on the other hand, have a higher TAM density only in the late stage. Quantification of **(D and D')** M1-like and **(E and E')** M2-like TAMs within the macrophage population, shows that opposing to eJag1^{KO} mice, which have more M1-like and less M2-like TAMs than the control counterparts, eJag1^{OE} mice have higher levels of M2-like TAMs, ate the expense of lower percentages of M1-like TAMs. Data are means ± SD (** $p<0.01$).

2.4.2. Expression of the Notch ligands Jag1 and Dll4 in endothelial cells directly affects macrophage polarization in vitro

Having shown that eJag1 modulation affected macrophage polarization in prostate tumors, we addressed whether this was occurring through direct macrophage-endothelial cell interactions. We isolated bone marrow CD11b^{+} monocytes from adult mice (**Figure 2.3A**) and placed them on top of human umbilical vein endothelial cells (HUVECs), previously plated and treated with neutralizing anti-human Jag1 and/or Dll4 antibodies (**Figure 2.3B**).

The data obtained in these experiments shows that, similar to what was observed in eJag1^{KO} mice, Jag1 neutralization in HUVECs decreased the total percentage of M2 macrophages. Since Dll4 has been shown to antagonize Jag1 in the angiogenic process (A. R. Pedrosa, Trindade, et al. 2015; Benedito et al. 2009; Gama-Norton et al. 2015), we addressed whether anti-Dll4 treatment in endothelial cells could have a distinct effect in macrophage polarization. Our results show that anti-Dll4 treatment exerts the same effect in macrophage polarization as anti-Jag1 treatment but a combination of both neutralizing

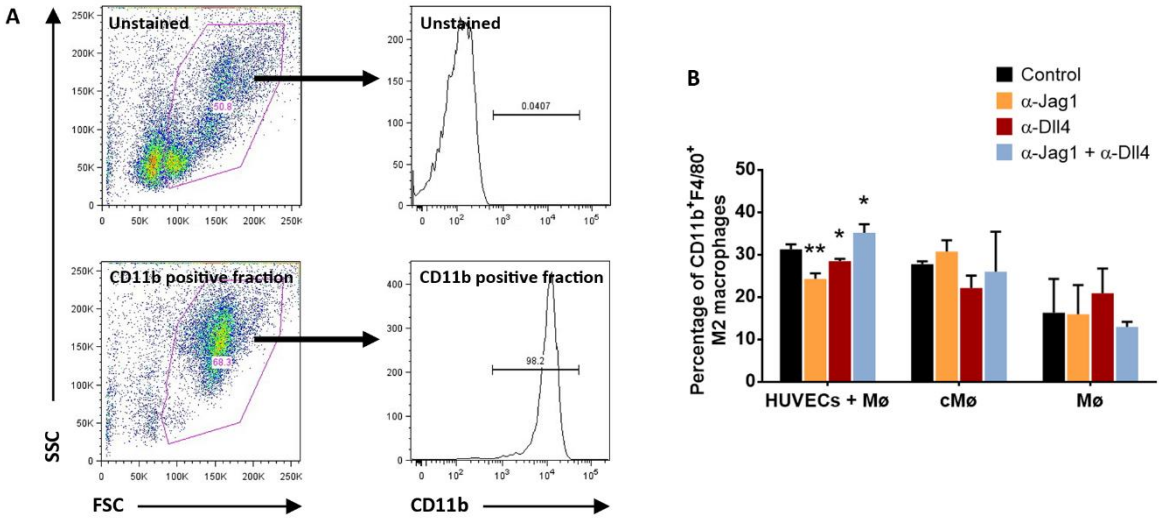

Figure 2.3. *In vitro* neutralization of the Notch ligands Jag1 and Dll4 affects macrophage polarization into the M2 state
(A) CD11b^{+} monocytes (Mø) were isolated from the bone marrow of adult mice and obtained with a purity of over 98%. **(B)** Monocytes were cultured on top of HUVECs previously treated with anti-Jag1 and/or anti-Dll4 neutralizing antibodies for 24h (HUVECs + Mø), on conditioned media from treated HUVECs (cMø) or with the neutralizing antibodies alone (Mø). Conditioned media and the neutralizing antibodies did not affect the percentage of M2 macrophages obtained after culturing monocytes for 4 days. However, anti-Jag1 and anti-Dll4 treated HUVECs induced a decrease in the polarization into M2 macrophages, whereas the combination of both neutralizing antibodies increased this percentage.

antibodies reverses this phenotype and induces an increase in macrophages in the M2 state. These alterations were absent when monocytes were cultured for the same period with conditioned media from anti-Jag1 and/or anti-Dll4 treated HUVECs (**Figure 2.3B**), suggesting that the polarization mechanism may require direct cell contact.

To exclude cross-reaction, monocytes were treated with the neutralizing antibodies alone. No differences were found in the percentage of M2-like macrophages when monocytes were incubated with anti-Jag1 and/or anti-Dll4 (**Figure 2.3B**), indicating that the results obtained upon HUVECs treatment are a result of the modulation of the Notch ligands specifically in endothelial cells.

2.4.3. eJag1 modulation affects the transcription profile of "angiocrine genes" in endothelial tumor-associated cells

To understand how endothelial Jag1 modulation affected the recruitment and/or activation of immune cells in the tumor site and macrophage polarization, we hypothesized that the observed alterations resulted from a modulation in the expression of endothelial specific factors, named "angiocrine factors" (Butler, Kobayashi, et al. 2010). Through the expression of these factors, endothelial cells establish an instructive vascular niche that directly modulates tumor cells (Butler, Kobayashi, et al. 2010; Franses et al. 2013) or other cells from the tumor microenvironment (He et al. 2012; Jurisic et al. 2010), regulating tumor initiation and progression.

To better understand the molecular mechanisms involved in immune cell recruitment and TAM polarization, we isolated $CD45^{-}Ter119^{-}CD31^{+}$ endothelial cells from prostate samples collected at the late (24w) stage of development and performed a qPCR analysis on Notch ligands and other angiocrine genes known to modulate hematopoietic cell mobilization and macrophage activation. Endothelial-specific gene transcription (**Figure 2.4**) suggests that endothelial Jag1 conditional knockout does not affect the expression of the Notch ligands *Dll1*, *Dll4* and *Jagged2*. Jag1 conditional overexpression, however, induces alterations in the expression of these genes in $CD31^{+}$ endothelial cells, significantly downregulating *Dll1* gene expression (≈67% decrease) and upregulating both *Dll4* and *Jagged2* (2.6 and 2.2-fold change, respectively). *Angpt2*, shown to be involved in the

establishment of direct macrophage-endothelial cell contacts (Baer et al. 2013), was upregulated in both mouse models (1.2 and 2.7 fold change in eJag1^{KO} and eJag1^{OE} mice, respectively). However, the expression of *Vcam1*, which encodes for an adhesion molecule known to play a critical role in inflammation by recruiting leukocytes to acute and chronic inflammatory sites (Luster et al. 2005; Osborn et al. 1989), is increased in eJag1^{KO} endothelial cells (1.6-fold change), but significantly decreased in eJag1^{OE} mice (79% decrease compared to the control counterparts). *Cxcl12* encodes for SDF1, which was previously shown to be involved in the retention of CXCR4^{+} monocytes close to the perivascular niche (Grunewald et al. 2006) and to regulate monocyte to macrophage differentiation (Sánchez-Martín et al. 2011). Our data suggest that *Cxcl12* transcription, similar to *Vcam1*, is increased in eJag1^{KO} endothelial cells but significantly decreases in Jag1-ovexpressing endothelial cells (68% lower transcription levels). These results reveal that endothelial-specific Jag1 modulation induces alterations in the expression profiles of angiocrine factors that regulate the recruitment and retention of hematopoietic cells in the tumor perivascular niche.

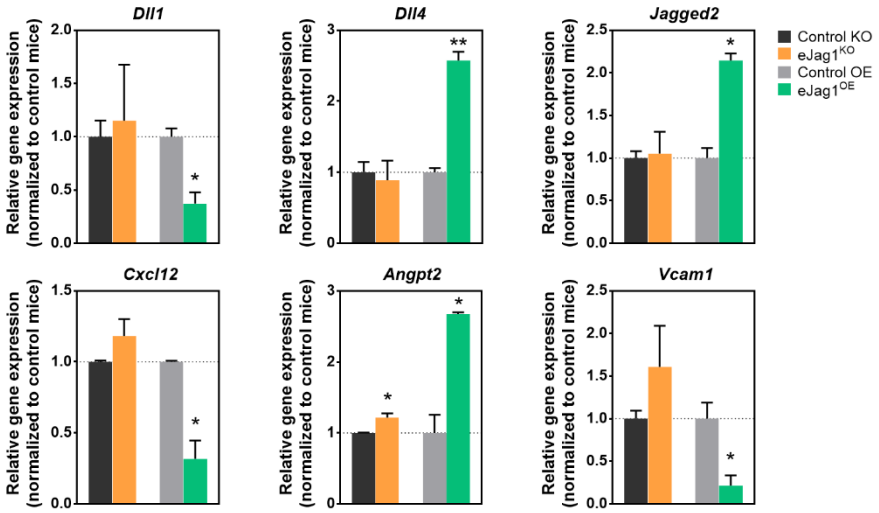

Figure 2.4. eJag1 modulates angiocrine gene expression in tumor-associated endothelial cells

CD31^{+} endothelial cells were isolated from prostate samples collected at 24 weeks (late stage) of age and angiocrine gene expression was assessed by qPCR. mRNA analysis showed that the expression of the genes encoding for the Notch ligands, *Dll1, Dll4* and *Jagged2*, is not affected in eJag1^{KO} endothelial cells, but eJag1^{OE} endothelial cells show higher levels of *Dll4* and *Jagged2*, and lower *Dll1* expression. Preliminary data suggest that CD31^{+} cells isolated form eJag1^{KO} have an increase in *Cxcl12* and *Vcam1* expression levels, whereas these genes are significantly downregulated in eJag1^{OE} endothelial cells. *Angpt2* is upregulated in both eJag1^{KO} and eJag1^{OE} endothelial cells. Data are means ± SD of 3 mice per experimental group (* $p<0.05$).

2.4.4. Modulation of eJag1 influences the transcription profile of tumor associated macrophages.

Depending on whether they are polarized into cytotoxic (M1) or tumor-promoting (M2) macrophages, TAMs express specific factors that give them either tumor stimulatory or inhibitory properties. However, the complexity of signals from the tumor microenvironment is such that intermediate states of activation are also found in tumors (Baer et al. 2013; Martinez & Gordon 2014).

To understand whether endothelial Jag1 could affect the expression pattern in TAMs, we performed a qPCR analysis on M1 ($CD11b^+/F4/80^+/MHCII^+/CD206^-$) and M2 ($CD11b^+/F4/80^+/MHCII^+/CD206^+$) macrophages isolated from prostate samples collected at 24 weeks of age (late stage) (**Figure 2.5**). Our results suggest that *Il6* and *Tnfa*, which code for cytokines involved in the pro-inflammatory M1 state, are not altered in M1 macrophages from either of the Jag1 mutant mice. However, they are both downregulated in eJag1^{KO} M2-derived macrophages and upregulated in M2 macrophages isolated from eJag1^{OE} prostate samples.

Cxcr4, which is involved in macrophage recruitment and in the interactions established between macrophages, tumor cells and other stromal cells (Beider et al. 2014; Baer et al. 2013; Mota et al. 2015), was found to have a much lower expression in M2 than in M1 macrophages, a difference observed both in eJag1^{KO} and eJag1^{OE} mice. However, no differences were found in *Cxcr4* transcription in M1 or M2 macrophages isolated from eJag1^{KO} or eJag1^{OE} mice, when compared to M1 or M2 cells isolated from the control counterparts.

mRNA analysis of *Vegfa*, the major angiogenesis regulator, suggests that M1 macrophages from eJag1^{KO} mice, but not from eJag1^{OE} mice, have a downregulation of *Vegfa* levels. On the other hand, *Vegfa* expression did not change in M2 macrophages isolated from eJag1^{KO} mice, but was downregulated in eJag1^{OE} mice. Regarding Notch receptors 1 and 2, our data show that *Notch1* transcription was not significant altered in M1 or M2 macrophages isolated from either of the Jag1 mutant mice. However, *Notch2*

was upregulated in M1 macrophages from eJag1^{KO} mice, and eJag1^{OE} mice exhibited a *Notch2* downregulation both in M1 and M2 macrophages.

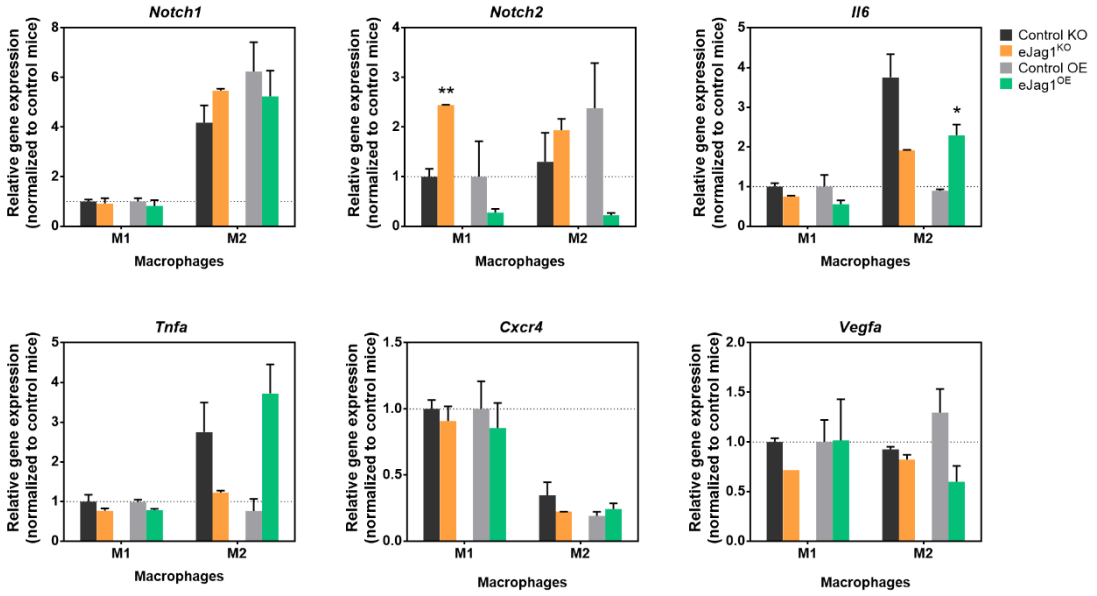

Figure 2.5. The expression profile of tumor associated macrophages is modified upon endothelial Jag1 modulation

Prostates were collected at the 24 weeks end-point, and M1 (CD11b$^+$/F4/80$^+$/MHCII$^+$/CD206$^-$) and M2 (CD11b$^+$/F4/80$^+$/MHCII$^+$/CD206$^+$) macrophages were sorted for specific gene transcription analysis. Gene transcript levels were normalized to the mRNA levels of M1 macrophages isolated from Control KO and Control OE for eJag1^{KO} and eJag1^{OE} mice, respectively. The expression pattern of M1 and M2 macrophages is very distinct, particularly for *Notch1*, *Il6*, *Tnfa* and *Cxcr4*. In M1 macrophages, only *Notch2* transcription is affected increasing in eJag1^{KO} mice and decreasing in eJag1^{OE} mice. In M2 macrophages from eJag1^{KO} mice, *Il6* and *Tnfa* are downregulated. In M2 macrophages from eJag1^{OE} mice, *Il6* and *Tnfa* are upregulated whereas *Notch2* and *Vegfa* expression levels are decreased. Data are means ± SD of 2-3 mice per experimental group (* p<0.05; ** p<0.01).

2.5. DISCUSSION

A large body of evidence suggests that tumor-associated macrophages (TAMs), the dominant leukocyte population found in the tumor microenvironment, actively participate in tumor initiation, growth and development. In the last decade, the interactions established between macrophages and endothelial cells have been characterized in detail, and the molecular players involved in these physical and functional relationships have been extensively studied (Mazzieri et al. 2011; De Palma et al. 2005; He et al. 2012; Lin et al. 2007; Owen & Mohamadzadeh 2013). The results presented in this Chapter describe the

effects of endothelial Jag1 modulation in the recruitment and polarization of tumor-associated macrophages.

We used well established mouse models to conditionally knockout or overexpress Jag1 in endothelial cells in mice that develop spontaneous prostate tumors (TRAMP). The transgene expression in TRAMP mice can be detected as early as 4 weeks of age and the initial epithelial hyperplasias are observed between 8 and 12 weeks of age. These lesions rapidly evolve into an adenocarcinoma and become invasive, metastasizing to the lymph nodes between 18 and 24 weeks of age, and to the lungs by 24 weeks of age (Gingrich et al. 1996; Gingrich & Greenberg 1996). In our study, mice were induced at 4 weeks of age, allowing for the establishment of a selective pressure in the tumor microenvironment as the tumor developed.

Pedrosa and colleagues recently described the effect of modulating endothelial Jag1 in tumor angiogenesis and its role in tumor development and progression. The authors found that eJag1 expression in TRAMP mice correlates with increased tumor vessel density, branching and perivascular maturation, affecting tumor vascular perfusion. Nonetheless, despite the increased perfusion and decreased extravasation observed in the tumors of mice overexpressing eJag1, the hypoxia extent was largely increased when compared to their control counterparts, possibly due to the increased proliferation and reduced apoptosis, which lead to increased oxygen consumption (A.-R. Pedrosa et al. 2015).

Our results show that endothelial Jag1 is also involved in macrophage recruitment to the tumor microenvironment and in their polarization into a pro-tumoral M2 state. We suggest that the differences observed in macrophage polarization may be “endothelial-dependent” since *in vitro* inhibition of Jag1 in HUVECs is enough to impair monocyte differentiation into M2 macrophages. However, tumors are complex systems where tumor and stromal cells establish numerous interactions (Udagawa & Wood 2010; Nakasone et al. 2012; He et al. 2012; Weis & Cheresh 2011; Barron & Rowley 2012) and, as such, we do not exclude that the observed phenotypes might also result from endothelial-dependent modulation of other cell types found in the tumor microenvironment or from the hypoxic microenvironment found in the tumors of eJag1 overexpressing mice. In fact, endothelial cells can modulate tumor cells behavior, increasing their invasiveness, proliferation and migration through the release of specific angiocrine factors (Brantley-Sieders et al. 2011;

Franses et al. 2013; Cao et al. 2014). Endothelial Jag1, particularly, induces the invasion and chemoresistance of B cell lymphoma cells, through the activation of the Notch2 receptor (Cao et al. 2014). Such endothelial-derived modifications in the tumor cells may be sufficient to modify the cytokine and growth factor release by the tumor cells and induce macrophage polarization towards an M2 state (Hollmén et al. 2015; Mantovani & Locati 2013; Meng et al. 2014).

Macrophages are known to infiltrate hypoxic tumor regions (Murdoch et al. 2004). This seems to be a particular feature of M2 macrophages, as pro-inflammatory M1 macrophages are located outside of the hypoxic environments (Laoui et al. 2014; Movahedi et al. 2010). Although these observations could lead us to speculate that increased hypoxia induced M2 polarization in our model, a recent study has demonstrated that rather than acting in macrophage differentiation and polarization, hypoxia regulates hypoxia-sensitive genes and angiogenic activity, particularly in M2 macrophages, fine-tuning their functions (Van Overmeire et al. 2014; Laoui et al. 2014).

Hypoxia-inducible factor (HIF)-1α is a key transcription factor that regulates hypoxia-inducible gene expression (Burke et al. 2002). Hypoxic induction of VEGF in TAMs renders them a pro-angiogenic phenotype and may justify the positive correlation between TAM infiltration and angiogenesis in a number of different tumors (Leek et al. 1996; Nishie et al. 1999; Koide et al. 2004; Hanada et al. 2000; Wu et al. 2012; Espinosa et al. 2011), including prostate carcinoma (Lissbrant et al. 2000). Hypoxia also upregulates CXCR4 in monocytes and macrophages in a HIF1α-dependent manner (Schioppa et al. 2003), increasing their chemotactic responsiveness to its specific ligand SDF1. Furthermore, HIF1α induces SDF1 expression in direct proportion to reduced oxygen tension in endothelial cells (Ceradini et al. 2004) and other cells from the tumor microenvironment, such as macrophages (Sánchez-Martín et al. 2011), increasing the migration and homing of circulating CXCR4-positive monocytes/macrophages to the hypoxic areas of the tumor. However, although our results showed that eJag1 expression correlated with an increase in macrophage density within the tumor, particularly M2 macrophages, which was consistent with the enhanced angiogenesis and tumor growth described in eJag1^{OE} animals by Pedrosa et al. (A.-R. Pedrosa et al. 2015), we did not find increased *Vegf* expression and *Cxcr4* was clearly

downregulated in M2 macrophages, compared to the classically activated macrophages, suggesting that hypoxia was not affecting macrophage gene expression as expected.

Although CD206, the macrophage mannose receptor, is a widely accepted M2 macrophage marker (Stein et al. 1992; Rőszer 2015), several studies have expanded the M1/M2 definitions, suggesting that activation exists on a spectrum and cannot be easily separated into defined groups (Biswas & Mantovani 2010; Martinez & Gordon 2014; Mantovani et al. 2005; Stout et al. 2005; Stout & Suttles 2004). Depending on the type of activation stimuli the macrophages are exposed to, they express different sets of markers that challenge the correct identification of a specific macrophage activation state (Murray et al. 2014), requiring the combination of several markers. This suggests that CD206 alone may not be sufficient to distinguish a defined macrophage subset, but only a group of differentially activated M2 macrophages, which may explain the downregulation of *Cxcr4* and the absence of *Vegf* upregulation in our subset of M2 macrophages. It may also justify the increase in *Tnfa* and *Il6* transcription in M2 macrophages, two genes that code for M1 markers and that play critical roles in their pro-inflammatory phenotype (Parameswaran & Patial 2010; Tanaka et al. 2014). Nonetheless, cytokines associated with M1 activation, such as TNFα and IL6, may be produced by M2 macrophages also (Sica & Mantovani 2012; Murray et al. 2014). IL6, particularly, has the ability to enforce the phenotype to which a macrophage has committed, enhancing IL1β and TNFα production by M1 macrophages, and conferring additional immunosuppressive bioactivities to M2 macrophages, such as IL10 production (Fernando et al. 2014; Mauer et al. 2014). Both TNFα and IL6 are potent angiogenic stimulators in inflammation (Tanaka et al. 2014; Ligresti et al. 2011). Despite their pro-inflammatory roles, which may explain the increase in B and T lymphocytes in the prostate samples of eJag1^{OE} mice, they have also been implicated in TAM-derived angiogenesis (Lee et al. 2006). Moreover, production of pro-inflammatory cytokines, including TNFα and IL6, by TAMs and other cells of the tumor microenvironment, sustains tumor growth and inhibits apoptosis (Lesina et al. 2011; Grivennikov et al. 2009; Fukuda et al. 2011; Bollrath & Greten 2009; Ribatti & Vacca 2009). Together, these observations suggest that TAMs may be contributing to the enhanced tumor growth and angiogenesis found in eJag1^{OE} mice through the production of inflammatory cytokines.

Modulation of tumor angiogenesis and vessel maturation led to alterations in the angiocrine gene transcription profile of endothelial cells, which ultimately may affect monocyte and macrophage recruitment. *Angpt2* upregulation in endothelial cells may be responsible for recruiting TAMs into tumors in eJag1^{OE} mice. Hypoxia-induced *Angpt2* expression can induce both active vascular remodeling (Huang et al. 2010) and the recruitment of Tie2-expressing macrophages to the tumor site (Murdoch et al. 2007; Lewis et al. 2007), which are highly pro-angiogenic and tend to cluster around vessels in some tumor models (De Palma et al. 2005). SDF1 is another potent monocyte chemoattractant that is upregulated in a HIF1α-dependent manner (Ceradini et al. 2004). The downregulation of *Cxcl12* expression in eJag1^{OE} mice may be caused by the increase in macrophage-derived TNFα, which inhibits SDF1 expression in endothelial cells (Salvucci et al. 2004). The opposite regulation is possibly occurring with VCAM1. Although TNFα upregulates *Vcam1*, which induces monocyte and cancer cell adhesion to endothelial cells (Sawa et al. 2007), an hypoxic environment was shown to inhibit TNFα-dependent VCAM1 induction (Cartee et al. 2012).

Given that the Notch signaling is known to play an important role in the development of hematopoietic and immune cells (Radtke et al. 2010), we speculated that endothelial-derived Notch signaling could be involved in modulating TAM polarization and transcription pattern. Notch signaling has already been demonstrated to play a critical role in the determination of M1 versus M2 polarization of macrophages. Compromised Notch signaling in macrophages lead to M2 polarization even in the presence of M1 inducers, whereas forced activation of Notch signaling increased the M1 response (Wang et al. 2010; Zhao et al. 2016). Endothelial Jag1 overexpression led to a modulation in the expression of the other Notch ligands in endothelial cells, upregulating *Dll4* and *Jag2* and downregulating *Dll1*. A similar expression pattern was found when mRNA from total prostate tumors from TRAMP mice was compared with normal prostates (A. R. Pedrosa, Graça, et al. 2015), suggesting that these ligands may be upregulated in malignancy. However, we were surprised by the higher endothelial *Dll4* expression levels in the tumors of these mice, considering that it functions as a negative regulator of tumor angiogenesis (Liu et al. 2014) and that eJag1^{OE} mice have a downregulation in endothelial *Dll4* in a setting of wound healing (A. R. Pedrosa, Trindade, et al. 2015). Furthermore, Dll4 was also shown to be

downregulated by TNFα signaling, whereas the opposite was found for both Jag1 and Jag2 (Benedito et al. 2009; Fernandez et al. 2008).

Despite the increase in endothelial Notch ligands, we observed higher levels of M2 macrophages in the tumors of eJag1^{OE} mice, suggesting that other mechanisms may be counteracting the Notch ligands role in macrophage polarization. One such mechanism may be the modulation of the transcription of Notch receptors in macrophages. We demonstrate that *Notch1* transcription is increased in M2 macrophages. Although this still does not explain why macrophages polarize into an M2 state in the presence of high levels of Notch ligands, Notch1 has been shown to be regulate a number of genes, and its increased expression might explain *Cxcr4* downregulation (Xie et al. 2013; Williams et al. 2008) and *Il6* and *Tnfa* upregulation (Outtz et al. 2010) in M2 macrophages, compared to M1 macrophages.

Interestingly, *Notch2* was downregulated in both M1 and M2 macrophages isolated from eJag1^{OE} prostate samples, whereas the opposite was found in eJag1^{KO}-derived macrophages. Considering that Notch signaling activation through a soluble Dll1 can induce cytokine specific macrophage apoptosis (Ohishi et al. 2000), we could hypothesize that the decrease in *Notch2* expression is a way for macrophages to evade apoptosis in eJag1^{OE} mice.

Together, our findings demonstrate for the first time that endothelial Jag1 modulation affects the recruitment and polarization of macrophages in the tumor microenvironment. Notably, higher levels of endothelial Jag1 not only increased the percentage of macrophages within the tumor, as it shifted the ratio of M1 versus M2 macrophages towards an M2 state, possibly through modulation of angiocrine genes, with consequent alterations in the macrophage expression patterns. Thus, these results suggest that endothelial cells may modulate the macrophage activation state towards a pro-angiogenic phenotype, in a positive feedback manner.

2.6. REFERENCES

Baer, C. et al., 2013. Reciprocal interactions between endothelial cells and macrophages in angiogenic vascular niches. *Experimental Cell Research*, 319(11), pp.1626–1634.

Bain, C.C. et al., 2014. Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nature immunology*, 15(10), pp.929–37.

Barron, D.A. & Rowley, D.R., 2012. The reactive stroma microenvironment and prostate cancer progression. *Endocrine-Related Cancer*, 19(6), pp.1–24.

Beider, K. et al., 2014. Multiple myeloma cells recruit tumor-supportive macrophages through the CXCR4/CXCL12 axis and promote their polarization toward the M2 phenotype. *Oncotarget*, 5(22), pp.11283–96.

Benedito, R. et al., 2009. The Notch Ligands Dll4 and Jagged1 Have Opposing Effects on Angiogenesis ¨. *Cell*, 137, pp.1124–1135.

Bingle, L., Brown, N.J. & Lewis, C.E., 2002. The role of tumour-associated macrophages in tumour progression: Implications for new anticancer therapies. *Journal of Pathology*, 196(3), pp.254–265.

Biswas, S.K. & Mantovani, A., 2010. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nature immunology*, 11(10), pp.889–896.

Bollrath, J. & Greten, F.R., 2009. IKK/NF-kappaB and STAT3 pathways: central signalling hubs in inflammation-mediated tumour promotion and metastasis. *EMBO reports*, 10(12), pp.1314–9.

Brantley-Sieders, D.M. et al., 2011. Angiocrine factors modulate tumor proliferation and motility through EphA2 repression of Slit2 tumor suppressor function in endothelium. *Cancer research*, 71(3), pp.976–87.

Burke, B. et al., 2002. Expression of HIF-1alpha by human macrophages: implications for the use of macrophages in hypoxia-regulated cancer gene therapy. *The Journal of pathology*, 196(2), pp.204–212.

Butler, J.M. et al., 2010. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nature reviews. Cancer*, 10(2), pp.138–146.

Cao, Z. et al., 2014. Angiocrine Factors Deployed by Tumor Vascular Niche Induce B Cell Lymphoma Invasiveness and Chemoresistance. *Cancer Cell*, 25(3), pp.350–365.

Cartee, T. V. et al., 2012. Hypoxia and hypoxia mimetics inhibit TNF-dependent VCAM1 induction in the 5A32 endothelial cell line via a hypoxia inducible factor dependent mechanism. *J Dermatol Sci*, 65(2), pp.86–94.

Ceradini, D.J. et al., 2004. Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nature medicine*, 10(8), pp.858–864.

Chanmee, T. et al., 2014. Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers*, 6(3), pp.1670–1690.

Cook-Mills, J.M. & Deem, T.L., 2005. Active participation of endothelial cells in inflammation. *J Leukoc Biol*, 77(4), pp.487–495.

Davidsson, S. et al., 2013. CD4 helper T cells, CD8 cytotoxic T cells, and FOXP3(+) regulatory T cells with respect to lethal prostate cancer. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*, 26(3), pp.448–55.

Erdag, G. et al., 2012. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. *Cancer Research*, 72(5), pp.1070–1080.

Espinosa, I. et al., 2011. CSF1 expression in nongynecological leiomyosarcoma is associated with increased

tumor angiogenesis. *American Journal of Pathology*, 179(4), pp.2100–2107.

Fernandez, L. et al., 2008. TNFα and Endothelial Cells Modulate Notch Signaling in the Bone Marrow Microenvironment during Inflammation. *Experimental Hematology*, 35(5), pp.545–558.

Fernando, M.R. et al., 2014. The pro-inflammatory cytokine, interleukin-6, enhances the polarization of alternatively activated macrophages. *PLoS ONE*, 9(4).

Franses, J.W. et al., 2013. Dysfunctional endothelial cells directly stimulate cancer inflammation and metastasis. *Int J Cancer*, 18(9), pp.1199–1216.

Fukuda, A. et al., 2011. Stat3 and MMP7 Contribute to Pancreatic Ductal Adenocarcinoma Initiation and Progression. *Cancer Cell*, 19(4), pp.441–455.

Fung, E. et al., 2007. Delta-like 4 induces Notch signaling in macrophages: Implications for inflammation. *Circulation*, 115(23), pp.2948–2956.

Gama-Norton, L. et al., 2015. Notch signal strength controls cell fate in the haemogenic endothelium. *Nature communications*, 6, p.8510.

Gingrich, J.R. et al., 1996. Metastatic Prostate Cancer in a Transgenic Mouse. *Cancer Research*, 56, pp.4096–4103.

Gingrich, J.R. & Greenberg, N.M., 1996. A transgenic mouse prostate cancer model. *Toxicologic Pathology*, 24(4), pp.502–504.

Gopisetty, A. et al., 2013. OX40L/Jagged1 Cosignaling by GM-CSF-Induced Bone Marrow-Derived Dendritic Cells Is Required for the Expansion of Functional Regulatory T Cells. *Journal of immunology (Baltimore, Md. : 1950)*, 190(11), pp.5516–25.

Gordon, S. & Martinez, F.O., 2010. Alternative activation of macrophages: Mechanism and functions. *Immunity*, 32(5), pp.593–604.

Grivennikov, S. et al., 2009. IL-6 and STAT3 are required for survival of intestinal epithelial cells and development of colitis associated cancer. *Cancer cell*, 15(2), pp.103–113.

Grunewald, M. et al., 2006. VEGF-induced adult neovascularization: Recruitment, retention, and role of accessory cells. *Cell*, 124(1), pp.175–189.

Gunderson, A.J. & Coussens, L.M., 2013. B cells and their Mediators as Targets for Therapy in Solid Tumors. *Experimental Cell Research*, 319(11), pp.1644–1649.

Hanada, T. et al., 2000. Prognostic value of tumor-associated macrophage count in human bladder cancer. *International Journal of Urology*, 7(7), pp.263–269.

He, H. et al., 2012. Endothelial cells provide an instructive niche for the differentiation and functional polarization of M2-like macrophages. *Blood*, 120(15), pp.3152–3162.

He, N. et al., 2014. Bone marrow vascular niche: home for hematopoietic stem cells. *Bone marrow research*, 2014, p.128436.

Hollmén, M. et al., 2015. Characterization of macrophage - cancer cell crosstalk in estrogen receptor positive and triple-negative breast cancer. *Scientific Reports*, 5(9188), pp.1–10.

Huang, H. et al., 2010. Targeting the ANGPT–TIE2 pathway in malignancy. *Nature Reviews Cancer*, 10(8), pp.575–585.

Jinushi, M. et al., 2011. Tumor-associated macrophages regulate tumorigenicity and anticancer drug responses of cancer stem/initiating cells. *Proceedings of the National Academy of Sciences of the United States of America*, 108(30), pp.12425–12430.

Jurisic, G. et al., 2010. Thymus cell antigen 1 (Thy1, CD90) is expressed by lymphatic vessels and mediates cell adhesion to lymphatic endothelium. *Experimental Cell Research*, 316(17), pp.2982–2992.

Kaplan-Lefko, P.J. et al., 2003. Pathobiology of autochthonous prostate cancer in a pre-clinical transgenic mouse model. *Prostate*, 55(3), pp.219–237.

Kiernan, A.E., Xu, J. & Gridley, T., 2006. The notch ligand JAG1 is required for sensory progenitor development in the mammalian inner ear. *PLoS Genetics*, 2(1), pp.27–38.

Kim, H. & Cantor, H., 2014. CD4 T-cell Subsets and Tumor Immunity: The Helpful and the Not-so-Helpful. *Cancer immunology research*, 2(2), pp.91–98.

Koide, N. et al., 2004. Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus. *The American journal of gastroenterology*, 99(9), pp.1667–1674.

Lai, E.C., 2004. Notch signaling: control of cell communication and cell fate. *Development (Cambridge, England)*, 131(5), pp.965–973.

Laoui, D. et al., 2014. Tumor hypoxia does not drive differentiation of tumor-associated macrophages but rather fine-tunes the M2-like macrophage population. *Cancer Research*, 74(1), pp.24–30.

Lee, C. et al., 2006. Tumor Necrosis Factor-α , Interleukin-8 and Interleukin-6 Are Involved in Vascular Endothelial Cell Capillary Tube and Network Formation Induced by Tumor-Associated Macrophages. *Journal of Cancer Molecules*, 2, pp.155–160.

Leek, R.D. et al., 1996. Association of Macrophage Infiltration with Angiogenesis and Prognosis in Invasive Breast Carcinoma. *Cancer Research*, 56(16), pp.4625–4629.

Lesina, M. et al., 2011. Stat3/Socs3 Activation by IL-6 Transsignaling Promotes Progression of Pancreatic Intraepithelial Neoplasia and Development of Pancreatic Cancer. *Cancer Cell*, 19(4), pp.456–469.

Lewis, C.E., De Palma, M. & Naldini, L., 2007. Tie2-expressing monocytes and tumor angiogenesis: Regulation by hypoxia and angiopoietin-2. *Cancer Research*, 67(18), pp.8429–8432.

Ligresti, G. et al., 2011. Macrophage-derived tumor necrosis factor-?? is an early component of the molecular cascade leading to angiogenesis in response to aortic injury. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 31(5), pp.1151–1159.

Lin, E.Y. et al., 2007. Vascular endothelial growth factor restores delayed tumor progression in tumors depleted of macrophages. *Molecular Oncology*, 1(3), pp.288–302.

Lissbrant, I. et al., 2000. Tumor associated macrophages in human prostate cancer- relation to clinicalpathological variables and survival. *International Journal of Oncology*, 17, pp.445–451.

Liu, Z. et al., 2014. Dll4-Notch signaling in regulation of tumor angiogenesis. *Journal of Cancer Research and Clinical Oncology*, 140(4), pp.525–536.

Luster, A.D., Alon, R. & von Andrian, U.H., 2005. Immune cell migration in inflammation: present and future therapeutic targets. *Nature Immunology*, 6(12), pp.1182–90.

Mantovani, A. & Locati, M., 2013. Tumor-associated macrophages as a paradigm of macrophage plasticity, diversity, and polarization lessons and open questions. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 33(7), pp.1478–1483.

Mantovani, A., Sica, A. & Locati, M., 2005. Macrophage polarization comes of age. *Immunity*, 23(4), pp.344–346.

Martinez, F.O. & Gordon, S., 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Reports*, 6(March), pp.1–13.

Mauer, J. et al., 2014. Signaling by IL-6 promotes alternative activation of macrophages to limit endotoxemia and obesity-associated resistance to insulin. *Nature immunology*, 15(5), pp.423–30.

Mazzieri, R. et al., 2011. Targeting the ANG2/TIE2 Axis Inhibits Tumor Growth and Metastasis by Impairing

Angiogenesis and Disabling Rebounds of Proangiogenic Myeloid Cells. *Cancer Cell*, 19(4), pp.512–526.

Meng, F. et al., 2014. Interaction between pancreatic cancer cells and tumor-associated macrophages promotes the invasion of pancreatic cancer cells and the differentiation and migration of macrophages. *IUBMB Life*, 66(12), pp.835–846.

Monvoisin, A. et al., 2006. VE-cadherin-CreER ^{T2} transgenic mouse: A model for inducible recombination in the endothelium. *Developmental Dynamics*, 235(12), pp.3413–3422.

Mota, J.M. et al., 2015. Post-sepsis state induces tumor-associated macrophage accumulation through CXCR4/CXCL12 and favors tumor progression in mice. *Cancer immunology research*, p.canimm.0170.2015.

Movahedi, K. et al., 2010. Different tumor microenvironments contain functionally distinct subsets of macrophages derived from Ly6C(high) monocytes. *Cancer Research*, 70(14), pp.5728–5739.

Murdoch, C. et al., 2007. Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. *Journal of immunology (Baltimore, Md. : 1950)*, 178(11), pp.7405–7411.

Murdoch, C., Giannoudis, A. & Lewis, C.E., 2004. Mechanisms regulating the recruitment of macrophages into hypoxic areas of tumors and other ischemic tissues. *Blood*, 104(8), pp.2224–2234.

Murray, P.J. et al., 2014. Macrophage Activation and Polarization: Nomenclature and Experimental Guidelines. *Immunity*, 41(1), pp.14–20.

Nakasone, E.S. et al., 2012. Imaging Tumor-Stroma Interactions during Chemotherapy Reveals Contributions of the Microenvironment to Resistance. *Cancer Cell*, 21(4), pp.488–503.

Nielsen, J.S. et al., 2012. CD20+ tumor-infiltrating lymphocytes have an atypical CD27 - memory phenotype and together with CD8+ T cells promote favorable prognosis in ovarian cancer. *Clinical Cancer Research*, 18(12), pp.3281–3292.

Nishie, a et al., 1999. Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas. *Clinical cancer research : an official journal of the American Association for Cancer Research*, 5(May), pp.1107–1113.

Ohishi, K. et al., 2000. Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. *Blood*, 95(9), pp.2847–54.

Ohno, S. et al., 2004. Correlation of histological localization of tumor-associated macrophages with clinicopathological features in endometrial cancer. *Anticancer Research*, 24(5 C), pp.3335–3342.

Osborn, L. et al., 1989. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell*, 59(6), pp.1203–1211.

Outtz, H.H. et al., 2011. Notch1 controls macrophage recruitment and Notch signaling is activated at sites of endothelial cell anastomosis during retinal angiogenesis in mice. *Blood*, 118(12), pp.3436–3439.

Outtz, H.H. et al., 2010. Notch1 deficiency results in decreased inflammation during wound healing and regulates vascular endothelial growth factor receptor-1 and inflammatory cytokine expression in macrophages. *Journal of immunology (Baltimore, Md. : 1950)*, 185(7), pp.4363–73.

Van Overmeire, E. et al., 2014. Hypoxia and tumor-associated macrophages: A deadly alliance in support of tumor progression. *Oncoimmunology*, 3(1), p.e27561.

Owen, J.L. & Mohamadzadeh, M., 2013. Macrophages and chemokines as mediators of angiogenesis. *Frontiers in Physiology*, 4 JUL(July), pp.1–8.

Page, D.B. et al., 2014. Immune modulation in cancer with antibodies. *Annual review of medicine*, 65(October 2013), pp.185–202.

De Palma, M. et al., 2005. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for

tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell*, 8(3), pp.211–226.

Parameswaran, N. & Patial, S., 2010. Tumor necrosis factor-α signaling in macrophages. *Critical reviews in eukaryotic gene expression*, 20(2), pp.87–103.

Pedrosa, A.R., Trindade, A., et al., 2015. Endothelial jagged1 antagonizes Dll4 regulation of endothelial branching and promotes vascular maturation downstream of Dll4/Notch1. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 35(5), pp.1134–1146.

Pedrosa, A.R., Graça, J.L., et al., 2015. Notch signaling dynamics in the adult healthy prostate and in prostatic tumor development. *Prostate*, 96, pp.80–96.

Pedrosa, A.-R. et al., 2015. Endothelial Jagged1 promotes solid tumor growth through both pro-angiogenic and angiocrine functions. *Oncotarget*, 6(27).

Pollard, J.W., 2004. Tumour-educated macrophages promote tumour progression and metastasis. *Nature reviews. Cancer*, 4(1), pp.71–78.

Radtke, F., Fasnacht, N. & MacDonald, H.R., 2010. Notch Signaling in the Immune System. *Immunity*, 32(1), pp.14–27.

Ribatti, D. & Vacca, A., 2009. The role of monocytes-macrophages in vasculogenesis in multiple myeloma. *Leukemia*, 23(9), pp.1535–6.

Rőszer, T., 2015. Understanding the Mysterious M2 Macrophage through Activation Markers and Effector Mechanisms. *Mediators of inflammation*, 2015, p.816460.

Roth, F. et al., 2012. Aptamer-mediated blockade of IL4Ra triggers apoptosis of MDSCs and limits tumor progression. *Cancer Research*, 72(6), pp.1373–1383.

Salvucci, O. et al., 2004. Evidence for the involvement of SDF-1 and CXCR4 in the disruption of endothelial cell-branching morphogenesis and angiogenesis by TNF-alpha and IFN-gamma. *Journal of leukocyte biology*, 76(1), pp.217–26.

Sánchez-Martín, L. et al., 2011. The chemokine CXCL12 regulates monocyte-macrophage differentiation and RUNX3 expression. *Blood*, 117(1), pp.88–97.

Sawa, Y. et al., 2007. Effects of TNF-alpha on leukocyte adhesion molecule expressions in cultured human lymphatic endothelium. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 55(7), pp.721–733.

Schioppa, T. et al., 2003. Regulation of the chemokine receptor CXCR4 by hypoxia. *The Journal of experimental medicine*, 198(9), pp.1391–402.

Schmidt, M. et al., 2008. The humoral immune system has a key prognostic impact in node-negative breast cancer. *Cancer Research*, 68(13), pp.5405–5413.

Sekine, C. et al., 2012. Differential regulation of osteoclastogenesis by Notch2 / Delta-like 1 and Notch1 / Jagged1 axes. *Arthritis Research & Therapy*, 14(2), p.R45.

Sica, A. & Mantovani, A., 2012. Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation*, 122(3), pp.787–795.

Spaner, D. & Bahlo, A., 2011. B Lymphocytes in Cancer Immunology. *Experimental and Applied Immunotherapy*, pp.1–442.

Stein, M. et al., 1992. Interleukin 4 potently enhances murine macrophage mannose receptor activity: a marker of alternative immunologic macrophage activation. *The Journal of experimental medicine*, 176(July), pp.287–292.

Stout, R.D. et al., 2005. Macrophages sequentially change their functional phenotype in response to changes

in microenvironmental influences. *Journal of immunology (Baltimore, Md. : 1950)*, 175(1), pp.342–349.

Stout, R.D. & Suttles, J., 2004. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *Journal of leukocyte biology*, 76(3), pp.509–13.

Sunamura, M. & Yagita, H., 2008. Neutralizing monoclonal antibody against human dll4.

Tanaka, T., Narazaki, M. & Kishimoto, T., 2014. IL-6 in Inflammation, Immunity, and Disease. *Cold Spring Harbor Perspectives in Biology*, 6(10), pp.a016295–a016295.

Tattersall, I.W. et al., 2016. In vitro modeling of endothelial interaction with macrophages and pericytes demonstrates Notch signaling function in the vascular microenvironment. *Angiogenesis*, 19(2), pp.201–215.

Udagawa, T. & Wood, M., 2010. Tumor-stromal cell interactions and opportunities for therapeutic intervention. *Current Opinion in Pharmacology*, 10(4), pp.369–374.

Wang, Y.C. et al., 2010. Notch signaling determines the M1 versus M2 polarization of macrophages in antitumor immune responses. *Cancer Research*, 70(12), pp.4840–4849.

Weis, S.M. & Cheresh, D.A., 2011. Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med*, 17(11), pp.1359–1370.

Williams, C.K. et al., 2008. Regulation of CXCR4 by the notch ligand delta-like 4 in endothelial cells. *Cancer Research*, 68(6), pp.1889–1895.

Wu, H. et al., 2012. Tumor-associated macrophages promote angiogenesis and lymphangiogenesis of gastric cancer. *Journal of Surgical Oncology*, 106(4), pp.462–468.

Xie, J. et al., 2013. Notch signaling regulates CXCR4 expression and the migration of mesenchymal stem cells. *Cellular immunology*, 281(1), pp.68–75.

Yu, P. & Fu, Y.-X., 2006. Tumor-infiltrating T lymphocytes: friends or foes? *Laboratory Investigation*, 86(3), pp.231–245.

Zanetti, M., 2015. Tapping CD4 T cells for cancer immunotherapy: the choice of personalized genomics. *Journal of Immunology (Baltimore, Md.: 1950)*, 194(5), pp.2049–2056.

Zhao, J.-L. et al., 2016. Forced Activation of Notch in Macrophages Represses Tumor Growth by Upregulating miR-125a and Disabling Tumor-Associated Macrophages. *Cancer Research*.

3.

ENDOTHELIAL DELTA-LIKE 4 MODULATES THE HEMATOPOIETIC SYSTEM AND HINDERS BONE MARROW RECOVERY FOLLOWING MYELOABLATION

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CONTENTS

3.1. ABSTRACT

Hematopoietic stem cell (HSC) niches are specialized microenvironments that regulate HSC self-renewal, differentiation and proliferation. The bone marrow vascular niche has emerged as an indispensable niche that supports hematopoiesis by regulating both HSC cell fate and the commitment of hematopoietic progenitors into specific lineages. The Notch signaling pathway plays an essential role in the development of HSCs and influences multiple lineage decisions of developing lymphoid and myeloid cells. Therefore, we sought to clarify the role of endothelial Delta-like 4 (Dll4) in hematopoiesis and BM recovery following irradiation. We used two conditional mouse models with endothelial-specific Dll4 (eDll4) loss- or gain-of-function (eDll4^{KO} and eDll4^{OE}) and analyzed them at steady state and 8 and 26 days after myeloablation induced by sub-lethal irradiation. At steady state, eDll4^{OE} mice showed a decrease in the relative percentage of circulating CD11b^{+} myeloid cells. eDll4^{KO} mice, however, had higher levels of B cells both in the bone marrow (BM) and in the periphery, decreased in overall BM cell content. eDll4^{KO} mice also showed higher content of multipotent progenitors (CFU-GEMM) but less granulocyte progenitors (CFU-G). Following irradiation, we show that eDll4 upregulation decreases platelet number by day 8, whereas eDll4 knockout induces a decrease in erythrocyte levels. Twenty-six days after sub-lethal irradiation, however, platelet and erythrocyte levels were restored to control levels, but eDll4 overexpression had induced myeloid and T cell differentiation in detriment of the B cell lineage, whereas the opposite was found for eDll4^{KO} mice. To finalize, eDll4 levels were shown to be negatively correlated with BM recovery. Together, our data reveal the hematopoietic effects of Dll4 modulation in the BM vasculature, suggesting that blocking Dll4 may be favorable in a setting of myeloablation.

3.2. INTRODUCTION

Establishment and maintenance of the blood system relies on self-renewing HSCs responsible for replenishing the pool of lineage-specific progenitor cells and effector blood cells that perform the physiological functions of the hematopoietic system, such as oxygen delivery, immunity and tissue remodeling (Orkin 2000). The ability of HSCs to self-renew

and differentiate throughout life has been widely accepted to be regulated by specialized bone marrow (BM) microenvironments, named HSC niches (Schofield 1978).

Osteoblasts and endothelial cells were the first to arise as major microenvironmental cells that regulate HSCs biology, comprising the osteoblastic and vascular niches, respectively (Kiel et al. 2005; Calvi et al. 2003). However, HSC niches are considerably more complex, being composed by several other BM stromal cells, as well as differentiated hematopoietic cells (Purton & Scadden 2012; Lo Celso & Scadden 2011). Furthermore, recent findings have suggested that osteoblasts may not have a direct effect in hematopoietic stem or progenitor cell maintenance (Sugiyama & Nagasawa 2012; Kiel et al. 2007; Kiel et al. 2009), and instead HSCs are differentially regulated by distinct perivascular niches (Kunisaki et al. 2013; Boulais & Frenette 2015; Morrison & Scadden 2014). The vascular niche is therefore critical for both HSC maintenance (Butler, Nolan, et al. 2010; Kimura et al. 2011; Ding et al. 2012; Kunisaki et al. 2013; Winkler et al. 2012; Sipkins et al. 2005; Himburg et al. 2012) and differentiation (Rafii et al. 1995; H. G. Kopp et al. 2005; Ding & Morrison 2013).

Following myelosuppression, caused by chemotherapy or radiation exposure, for example, endothelial cells are required for hematopoietic reconstitution and recovery (H. G. Kopp et al. 2005; Hooper et al. 2009; Li et al. 2010; Salter et al. 2009; Chute et al. 2007). In fact, endothelial cells not only regenerate and replenish the HSC population after myeloablation (Ding et al. 2012; Kobayashi et al. 2010; Butler, Nolan, et al. 2010; Doan et al. 2013), as they are also involved in the differentiation of the hematopoietic progenitors (Avecilla et al. 2004; Hamada et al. 1998).

The Notch signaling pathway seems to be involved in a number of cell fate decisions in the hematopoietic system, regulating not only HSCs biology but also the lineage-specific commitment of hematopoietic progenitors (J. Liu et al. 2010; Radtke et al. 2013; Radtke et al. 2010). The Notch ligand Delta-like 4 (Dll4), particularly, has been shown to regulate both HSC fate (Lahmar et al. 2008; Dando et al. 2005; Karanu et al. 2001; Lauret et al. 2004) as well as the commitment and maturation of the lymphoid and myeloid lineages (Koch et al. 2008; Hozumi et al. 2008; Mohtashami et al. 2010; Coste et al. 2013; Dorsch et al. 2002; Poirault-Chassac et al. 2010; Dando et al. 2005; Laranjeiro et al. 2012). Although expression of the Notch ligands Jag1 and Jag2 by endothelial cells was found to promote proliferation

and prevent exhaustion of HSCs (Butler, Nolan, et al. 2010), the contribution of other endothelial-derived Notch ligands in hematopoiesis has not been addressed.

Our study characterized for the first time the effect of directly modulating endothelial Dll4 in hematopoiesis and BM recovery following myeloablation. Using endothelial-specific Dll4 loss- and gain-of-function mouse mutants, we show that eDll4 depletion favors BM recovery after myelosuppression and induces B lymphocyte expansion in the BM but not mobilization into the periphery. Furthermore, eDll4 also affects platelet and erythrocyte levels but our results suggest different endothelial subsets may be involved in the regulation of each lineage.

3.3. METHODS

3.3.1. Animal genotyping

Mice genotyping was carried out using tail snips or ear punch biopsies digested at 55ºC, overnight with constant shaking, in a solution of Laird's buffer (1M Tris HCl pH 8.5, 0,5M EDTA, 20% SDS, 5M NaCl) containing 100µg/mL Proteinase K (Sigma). Following digestion, hair and debris were discarded by spinning tubes at 13.000 rpm for 5 minutes. The supernatant was collected into a fresh tube and DNA was precipitated by adding an equal volume of isopropanol. With a clean, sterile, micropipette tip, the long thread-like precipitate of DNA was transferred into a new tube and dissolved in ddH_2O.

Mice were genotyped using 4µL of DNA solution in a mix containing 250µM dNTPs, 0,35µM of forward and reverse primers (**Table 3.I**), 1U Taq Polimerase (Invitrogen) and 5mM $MgCl_2$ when primers for $Dll4^{lox/lox}$ were used or 2,5mM $MgCl_2$ for the VE-Cadherin-Cre-ER^{T2}, Dll4-Tet-O7 and Tie2-rtTA primer pairs. The PCR program was similar for all primers pairs, and 60ºC was found to the optimal annealing temperature. Expected product sizes are depicted in **Table 3.I**.

Table 3.I. Primers used for genotyping.

Genotype	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size
$Dll4^{lox/lox}$	GTGCTGGGACTGTAGCCACT	TGTTAGGGATGTCGCTCTCC	455 bp
VE-Cadherin-Cre-ER^{T2}	CCAGCTAAACATGCTTCATC	CGCTCGACCAGTTTAGTTAC	350 bp
Dll4-Tet-O7	ATCCACGCTGTTTTGACCTC	GTGGAGACATTGCCAAAGGT	500 bp
Tie2-rtTA	AAGTCATTCCGCTGTGCTCT	GTCTCAGAAGTGGGGGCATA	200 bp

3.3.2. Animal experiments

All experimental animal procedures performed in this study were previously approved by the Instituto de Medicina Molecular Ethics Committee and executed in strict compliance to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). The generation of both $Dll4^{lox/lox}$*VE-Cadherin-Cre-ERT2 ($eDll4^{KO}$) and of Dll4-Tet-O7*Tie2-rtTA ($eDll4^{OE}$) has been previously described (Trindade et al. 2012; Trindade et al. 2008). Briefly, to obtain the loss-of-function mice, $Dll4^{lox/lox}$ mice (in which the coding region for the first three exons of Dll4 is flanked by loxP sites) (Koch & Radtke 2011) were crossed with VE-Cadherin-CRE-ERT2 mice (Monvoisin et al. 2006), generating a conditional knockout mice ($eDll4^{KO}$; genetic background of C57/B6J mice). To deplete Dll4 specifically in endothelial cells, mice were induced with tamoxifen IP injection (50 mg/kg/day in castor oil, Sigma) for 5 consecutive days, starting 3 days after sub-lethal irradiation (300 rad) (see **Figure 3.1A** for the detailed experimental setup). Since Cre recombination occurs preferentially in S-phase (Hashimoto et al. 2008), we decided to induce the animals after irradiation to make sure that the endothelial cells were dividing (cycling) at the time of induction. Control mice (Control KO) had the same genotype but were injected with castor oil alone.

The gain-of-function Dll4 mutants were obtained by crossing Tet-O7-Dll4 mice with heterozygous Tie2-rtTA mutant mice (Trindade et al. 2008), generating a conditional Dll4 overexpressing mouse mutant ($eDll4^{OE}$; genetic background of FVB/NJ mice). To induce the Dll4 overexpression under the control of the Tie2 promotor, $eDll4^{OE}$ mice were induced through the administration of 2 mg/mL doxycycline (Sigma) in drinking water, with 2.5% sucrose, starting 7 days before irradiation and throughout the entire experiments (**Figure 3.1B**). The control mice (Control OE) had the same gain-of-function genotype, but were given only sucrose in their drinking water. All mice models - $eDll4^{KO}$, $eDll4^{OE}$ and respective

controls - were sacrificed at 8 and 26 days after sub-lethal irradiation. Non-irradiated mice were sacrificed at the same time-point as those sacrificed 26 days following irradiation, regarding the time of induction.

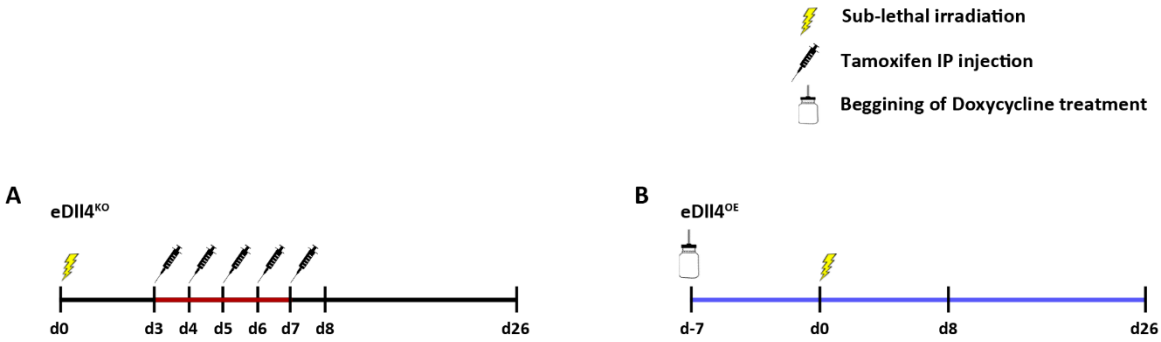

Figure 3.1. Experimental setup

(A) eDll4^{KO} mice were irradiated at day 0 and induced with tamoxifen starting at day 3 for 5 consecutive days. **(B)** eDll4^{OE} mice were induced with doxycycline in their drinking water 7 days before sub-lethal irradiation. Both experimental groups were sacrificed at days 8 or 26 following irradiation. Non-irradiated animals were induced at the same time-points and sacrificed at the equivalent of 26 days after irradiation.

3.3.3. Sample collection

Peripheral blood samples were collected by cardiac puncture onto EDTA-coated tubes (Multivette 600, Sarstedt), and complete blood counts were performed using a PocH-100iV Hematology Analyzer (Sysmex).

Tibia BM cells were flushed out with PBS 2mM EDTA and the total BM cell count was assessed using a Burker hemocytometer (Blau Brand). Cell were then centrifuged at 1200 rpm for 5 minutes and both PB and BM cells were collected for FACS analysis.

3.3.4. *In vitro* colony forming assay

To sort HSPCs, BM cells were flushed out from the long bones with PBS 2mM EDTA, treated with red blood cell lysis buffer (BioLegend) for 15 min in the dark, and enriched for the Lineage negative population by magnetic cell sorting using a Lineage antibody cocktail coupled to magnetic beads (MACS system, Miltenyi Biotec). Lineage$^-$ cells were collected and transferred into a new column. Again by magnetic cell sorting, Lineage$^-$Sca$^+$ cells were

obtained using a Sca-1 MicroBead Kit (FITC) containing anti-FITC magnetic beads (MACS system, Miltenyi Biotec).

Lineage^{-}Sca^{+} cells (10^5 cells) were plated onto a semi-solid cytokine-supplemented methylcellulose medium (MethoCult GF M3434) (Stemcell Technologies). Each colony formed in this semi-solid medium is single-cell derived and represents the identity of the original progenitor cell (Bradley & Metcalf 1966; Coulombel 2004). The resulting colonies were scored after 2 weeks of culture, according to the manufacturer's instructions.

3.3.5. Flow cytometry

Bone marrow and peripheral blood cells were treated with Red Blood Cell Lysis Buffer (Biolegend) for 15 minutes in the dark and were then stained for Anti-Lineage Biotin (CD5, CD45R (B220), CD11b, Anti-Gr-1 (Ly-6G/C), 7-4, and Ter-119) (Miltenyi Biotec), anti-Sca1 (E13-161.7) fluorescein isothiocyanate (FITC), anti-CD117 (2B8) allophycocyanin (APC), anti-CD19 (1D3) PE-Cyanine 7 (PE/Cy7), anti-CD45R (RA3-6B2) PE, anti-CD11b (M1/70) FITC (all from BD Biosciences), anti-CD3e (145-2C11) PE (eBiosciences), anti-CD4 (GK1.5) Pacific Blue and anti-CD8 (53-6.7) PerCP (BioLegend). Dead cells and debris were excluded by FSC, SSC and 7AAD (7-Amino-Actinomycin D) cell viability solution (BD Biosciences) staining profiles. Flow cytometric analyses were carried out using an LSR Fortessa flow cytometer equipped with FACS Diva 6.2 Software (BD Biosciences). Data were analyzed with a FlowJo 9.8.2 Software.

3.3.6. Bone marrow transplants

Control KO and Dll4^{KO} donor and recipient mice were all induced at the same time as described in Section 3.3.2. Twenty-six days after induction, recipient mice were lethally irradiated (900 rad) and subjected to BM transplants (BMT) 24 hours later. BM cells from previously induced and non-induced animals were collected from the long bones and mononuclear cells for BMT were obtained with LymphoSep®-Lymphocyte Separation Medium (MP Biochemichals). Tubes were centrifuged for 30 minutes at 1800 rpm at room temperature and the interface containing the BM mononuclear cells was collected. A total

of $2x10^6$ cells were injected intravenously and mice were sacrificed 25 days after BMT (26 days following lethal irradiation).

3.3.7. Statistical analysis

Data processing was carried out using Graph Pad Prism 6 Software and statistical analysis was performed using unpaired two-tailed Student's t test. Results are expressed as mean ± standard deviation. P values of <0.05 were considered statistically significant.

3.4. RESULTS

3.4.1. Endothelial Dll4 affects bone marrow cell content and the colony forming potential of eDll4^{KO} hematopoietic stem and progenitor cells

Notch signaling and the BM vascular niche have long been recognized for their roles in HSC maintenance and hematopoietic development (Karanu et al. 2000; Butler, Nolan, et al. 2010; Varnum-Finney et al. 1998; Stier et al. 2002; Duncan et al. 2005; Calvi et al. 2003; Radtke et al. 2010; Kobayashi et al. 2010; He et al. 2014). Therefore, we asked whether modulation of endothelial-specific Dll4 (eDll4) could affect BM function and hematopoiesis. With that in mind, two conditional and inducible mouse models were used: an endothelial Dll4 knock-out model (eDll4^{KO}) and an endothelial Dll4 overexpression model (eDll4^{OE}) (described in Section 3.3.2).

Mice were induced at 8 weeks of age and sacrificed approximately 1 month later to guarantee that the endothelial cells in which Dll4 was modulated had enough time to exert their effect in other BM stromal cells and cells from the hematopoietic compartment. Indeed, we found that the BM cellular content of eDll4^{KO} mice – but not eDll4^{OE} mice – was decreased, without affecting mice body weight, indicating that eDll4 is required to maintain normal BM cell numbers (**Figure 3.2A**). Nonetheless, we did not observe a significant variation in the percentage of Lin$^-$Sca$^+$c-kit$^+$ hematopoietic stem and progenitor cells (HSPCs) in neither mutant mice (**Figure 3.2B**) or in the colony-forming unit potential of eDll4^{KO}-derived HSPCs (**Figure 3.2C**). Such assays allowed us to identify and count single-

cell derived colonies, representing either multipotent (CFU-granulocyte-erythrocyte-macrophage-megakaryocyte, CFU-GEMM) or monopotent (CFU-monocyte, CFU-M; CFU-granulocyte, CFU-G; or bursting forming units-erythrocyte, BFU-E) progenitors. Our data show Dll4 conditional knockout significantly increased CFU-G colony numbers and decreased the multipotent potential (CFU-GEEM), without affecting in the monocyte and erythrocyte colony-forming unit capacity (CFU-M of BFU-E) (**Figure 3.2D**).

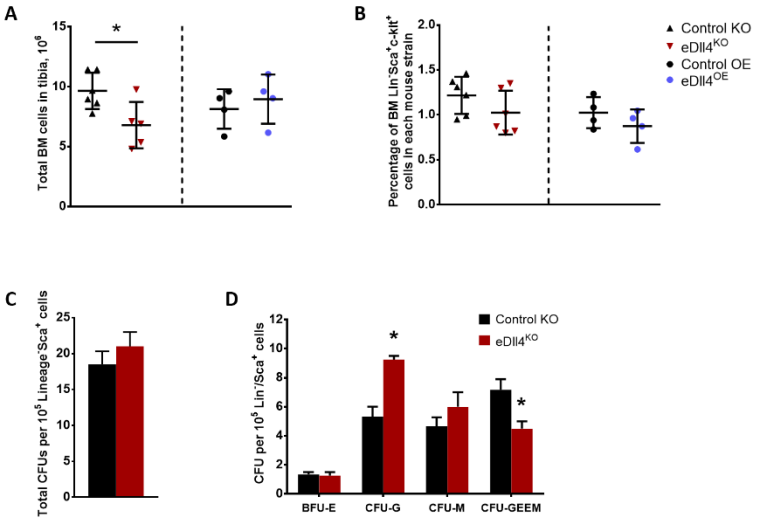

Figure 3.2. Endothelial Dll4 knockout affects BM cellularity and the CFU potential of HSPCs

(A) Total BM cell count shows that eDll4 modulation does not modify BM cellularity but the **(B)** quantification of $Lin^{-}Sca^{+}c\text{-}kit^{+}$ stem and progenitor cells did not reveal a significant alteration upon eDll4 modulation. **(C)** Colony-forming unit counts from methylcellulose culture of 10^5 $eDll4^{KO}$-derived $Lin^{-}Sca^{+}$ HSPCs reveal that eDll4 knockout does affect the stem cell capacity of these mice but **(D)** particularly increases granulocyte (CFU-G) colonies and decreased multipotent (CFU-GEEM) colonies, without affecting erythroid (BFU-E) or monocyte (CFU-M) potential. Data are means ± SD of at least 3 mice (* $p<0.05$).

3.4.2. Endothelial specific Dll4 modulates bone marrow and peripheral blood hematopoietic content

Considering that the BM cellularity and CFU potential were modified, particularly in $eDll4^{KO}$ mice, we sought to determine whether the myeloid and lymphoid composition of both BM and peripheral blood (PB) were also affected upon eDll4 modulation. Our data shows that endothelial-specific Dll4 knockout or overexpression did not affect the BM myeloid ($CD11b^{+}$) or the T lymphoid ($CD3^{+}$; $CD3^{+}CD4^{+}$; $CD3^{+}CD8^{+}$) compartments (**Figure 3.3A, C, D and E**). B lymphocyte percentage was also unaltered in $eDll4^{OE}$ BM, but showed a 2-fold

increase in the BM of eDll4^{KO} mice (**Figure 3.3B**). An identical phenotype was found in circulating B cells, which significantly increased from 28,76% ± 8,28 in Control KO mice to 43,33% ± 5.93 in eDll4^{KO} mice, but did not change in eDll4^{OE} mice, compared to their

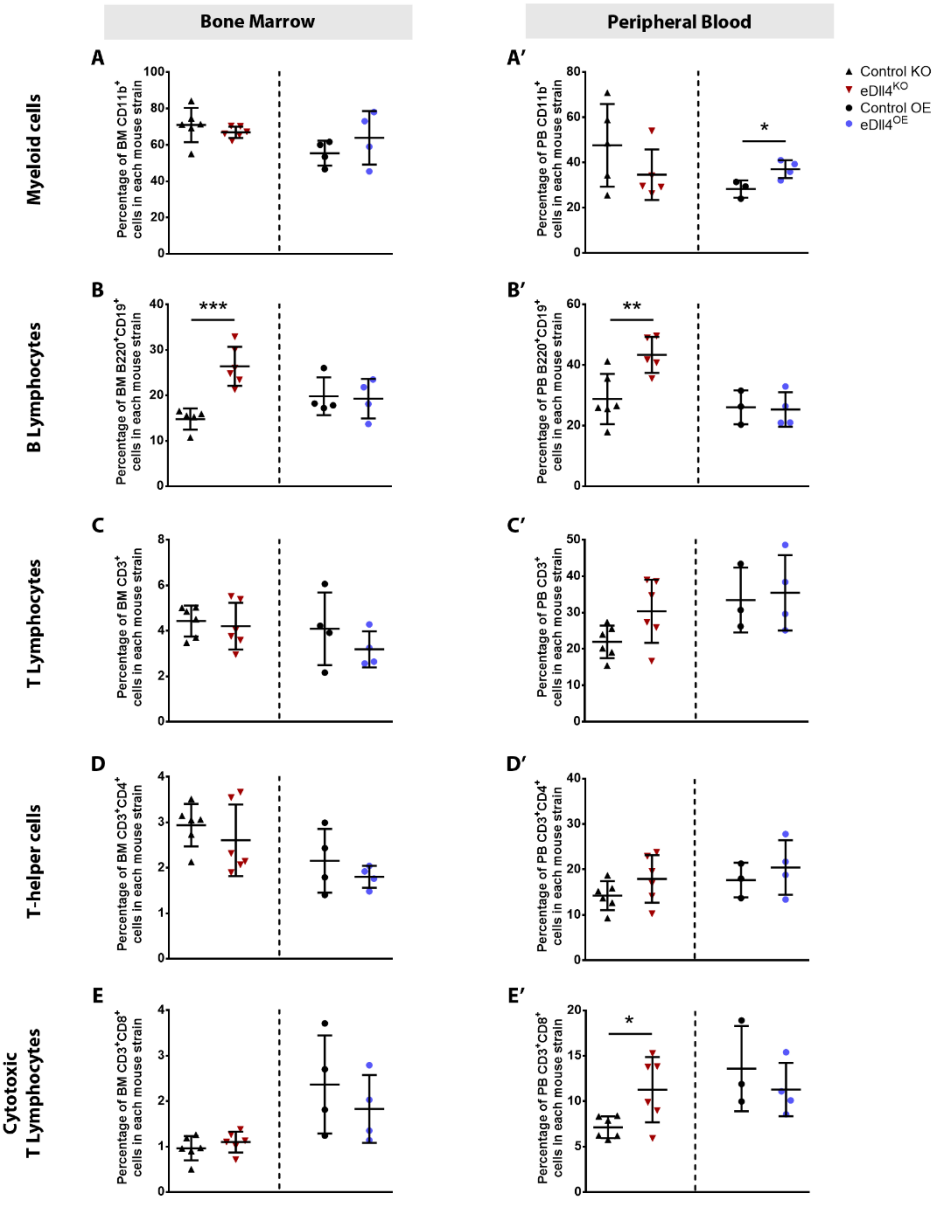

Figure 3.3. eDll4 modulation affects BM and PB lymphoid and myeloid content

Quantification of BM **(A-E)** hematopoietic polutations showed that eDll4 does not significantly affect the the **(A)** myeloid, **(C)** total CD3 T lymphocyte, **(D)** helper T cells or **(E)** cytotoxic T cells percentage in the BM. Analysis of the percentage of **(B)** B220^{+}CD19^{+} B cells showed a significant increase in eDll4^{KO} but not in eDll4^{OE} mice. Circulating cells quantification **(A'-E')** showed that (A') circulating myeloid cells were increased in eDll4^{OE} mice, but showed no alterations in eDll4^{KO} mice. Similar to what is described in the BM **(B')** B cells in circulation were found to be increased in eDll4^{KO} but not in eDll4^{OE} mice and **(C')** total T cells and **(D')** helper T cells remained unchanged in circulation. **(E')** Cytotoxic T cells were increased eDll4^{KO} but not in eDll4^{OE} mice. Data are means ± SD of at least 3 mice (* p<0.05, ** p<0.01, *** p<0.001).

control counterparts (**Figure 3.3B'**).

Although BM myeloid content did not show any differences upon eDll4 modulation, PB CD11b^{+} cells were found to be significantly increased in eDll4^{OE} mice (28,27% ± 3.83 and 37,08 ± 3,94 in Control OE and eDll4^{OE} mice, respectively), whereas eDll4^{KO} circulating myeloid cells remained unchanged (**Figure 3.3A'**). In line with the data obtained for BM T cells, circulating T lymphocytes and CD3^{+}CD4^{+} T helper cells did not reveal any alterations in any of the mice mutants (**Figure 3.3C' and D'**). However, eDll4^{KO} - but not in eDll4^{OE} mice – showed a 1.6 fold increase in cytotoxic CD3^{+}CD8^{+} T lymphocytes (**Figure 3.3E'**).

As shown in **Figure 3.4A**, eDll4 modulation did not affect erythrocyte counts, the hematocrit or the concentration of hemoglobin in each cell, but overexpressing – and not knocking out - eDll4 increased hemoglobin concentration (**Figure 3.4A**). Leukocyte number and the ratio of small over large white blood cells were not affected in either mutant mice (**Figure 3.4B**), which was also observed for platelet number and mean platelet volume (**Figure 3.4C**). Taken together, eDll4 modulation did not cause general changes in the blood parameters with the exception of hemoglobin concentration.

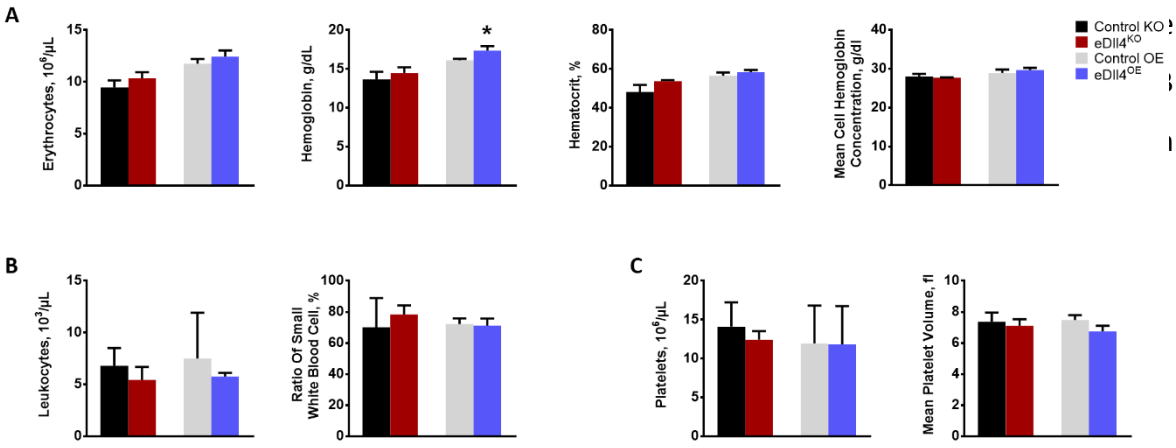

Figure 3.4. eDll4 does not affect the levels of erythrocytes, leukocytes or platelets in circulation

(A) Erythrocyte counts, hemoglobin, hematocrit and mean cell hemoglobin concentration were assessed by complete blood counts, showing that eDll4 only affects hemoglobin concentration in eDll4^{OE} mice, but none of the other parameters were affected in none of the mutant mice. **(B)** Leukocytes, and the ratio of small over large white blood cells as well as **(C)** platelet levels and mean platelet volume were also assessed, revealing no differences in either mutant mice from the respective control. Data are means ± SD of at least 3 mice (* $p<0.05$).

3.4.3. eDll4 modulation affects BM recovery and hematopoiesis after myeloablation

Bone marrow endothelial cells are major regulators of hematopoietic recovery following an acute injury to the bone marrow microenvironment, such as chemotherapy or irradiation, regulating HSC regeneration and differentiation (Ding et al. 2012; Butler, Kobayashi, et al. 2010; Butler, Nolan, et al. 2010) and protecting HSPCs from the irradiation damages (Doan et al. 2013). Hence, we next aimed to understand whether the modifications in the BM vascular compartment caused by eDll4 modulation affected BM hematopoietic recovery following sub-lethal total body irradiation.

A previous report from Remédio et al. had shown that, in a BM transplant setting, anti-Dll4 treatment of donor BM improved the hematopoietic recovery of lethally irradiated mice (Remédio et al. 2012). To determine the hematopoietic damage caused by irradiation and whether eDll4 affected BM recovery, the changes in BM cellularity were studied 8, 15 and 26 days after myeloablation (**Figure 3.5**). Our results revealed that sub-lethal irradiation induced a marked decrease in total BM cells at day 8 and reached a minimum at day 15. Nonetheless, BM cellularity in both time-points was similar in both mutants and their control counterparts.

Although autologous BM recovery has been described to occur less than 28 days after irradiation (Banna et al. 2004; Champlin et al. 2000), by day 26 post-irradiation, Control KO and eDll4^{KO} had only reached about 57% and 73% of the total BM cells found in non-irradiated mice, respectively (**Figure 3.5A**). Similarly, although Control OE mice had reached

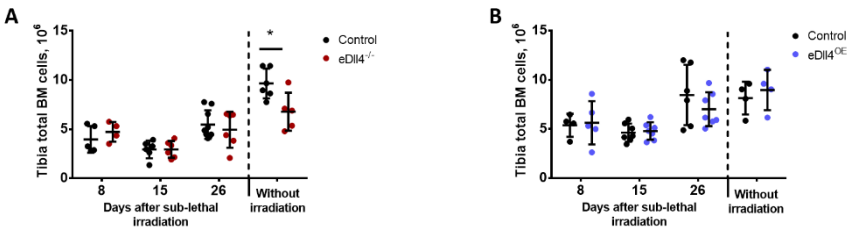

Figure 3.5. Endothelial Dll4 affects BM recovery following myeloablation

Total BM cell counts from eDll4 mutant mice following sub-lethal irradiation (300 rad) show that both mouse mutants had a marked decrease in BM cellularity by day 8, which reached a minimum by day 15 after irradiation, irrespective of eDll4 levels. By day 26 after irradiation **(A)** eDll4^{KO} mice showed BM cell counts closer to the non-irradiated animals than Control KO mice. **(B)** By day 26 post-irradiation, Control OE mice reached the total BM cell values of non-irradiated mice, but eDll4^{OE} mice had lower levels than the non-irradiated counterparts. Data are means ± SD of at least 4 mice (* $p<0.05$).

the values of non-irradiated mice by day 26 post-irradiation, eDll4^{OE} mice had 73% of the total tibia cells found in eDll4^{OE} mice that had not been exposed to irradiation (**Figure 3.5B**). Our data thus suggests that eDll4 negatively affects BM recovery, particularly in later time-points.

Having shown that eDll4 modulates BM recovery following sub-lethal irradiation, we next asked whether the percentage of the major hematopoietic lineages also changed. To do so, we analyzed mice 8 and 26 days after sub-lethal irradiation, accounting for an early and late stage of BM recovery. We observed that by day 8 post-irradiation, eDll4 modulation did not affect the stem cell ($Lin^-Sca^+c\text{-}kit^+$) or myeloid ($CD11b^+$) compartments (**Figure 3.6A and B**). However, both eDll4^{KO} and eDll4^{OE} presented quantitative alterations in the BM lymphoid compartment (**Figure 3.6C and D**). Particularly, eDll4^{KO} showed a decrease in $B220^+$ B cells and no visible alterations in $CD3^+$ T cells, whereas eDll4^{OE} mice exhibited an increased in B cell percentage and a decrease in the T cell compartment, which was due to a decrease in $CD3^+CD4^+$ T helper cells (**Figure 3.6E**). Moreover, although eDll4^{KO} mice did not present alterations in total BM T cells, they had a significant increase in $CD3^+CD8^+$ cytotoxic T cells (**Figure 3.6F**).

To address if these changes in the BM resulted in modulation of peripheral blood (circulating) cells, next we performed complete blood counts (CBCs) and a flow cytometry analysis of the PB content (**Figure 3.7**). Our data shows that eDll4 modulation did not affect erythrocyte counts, hemoglobin, the hematocrit or hemoglobin concentration (**Figure 3.7A**). Leukocyte number and the ratio of small over large white blood cells were also similar between eDll4^{KO}, eDll4^{OE} and the respective controls (**Figure 3.7B**). In contrast, overexpressing - but not knocking out - eDll4 caused a decrease in platelet levels. Mean platelet volume was not affected in either mutant mouse line (**Figure 3.7C**). Taken together, eDll4 modulation did not cause general changes in the blood parameters with the exception of platelets. However, a lineage-specific analysis of the circulating populations revealed that eDll4^{KO} had an increase in circulating myeloid cells (**Figure 3.7D**) and a decrease in the peripheral blood lymphoid content (B and T-helper cells, but not cytotoxic T lymphocytes) (**Figure 3.7E, F, G and H**). Overexpressing eDll4, however, only exerted an effect on circulating cytotoxic T cells, which were significantly decreased compared to Control OE mice (**Figure 3.7H**).

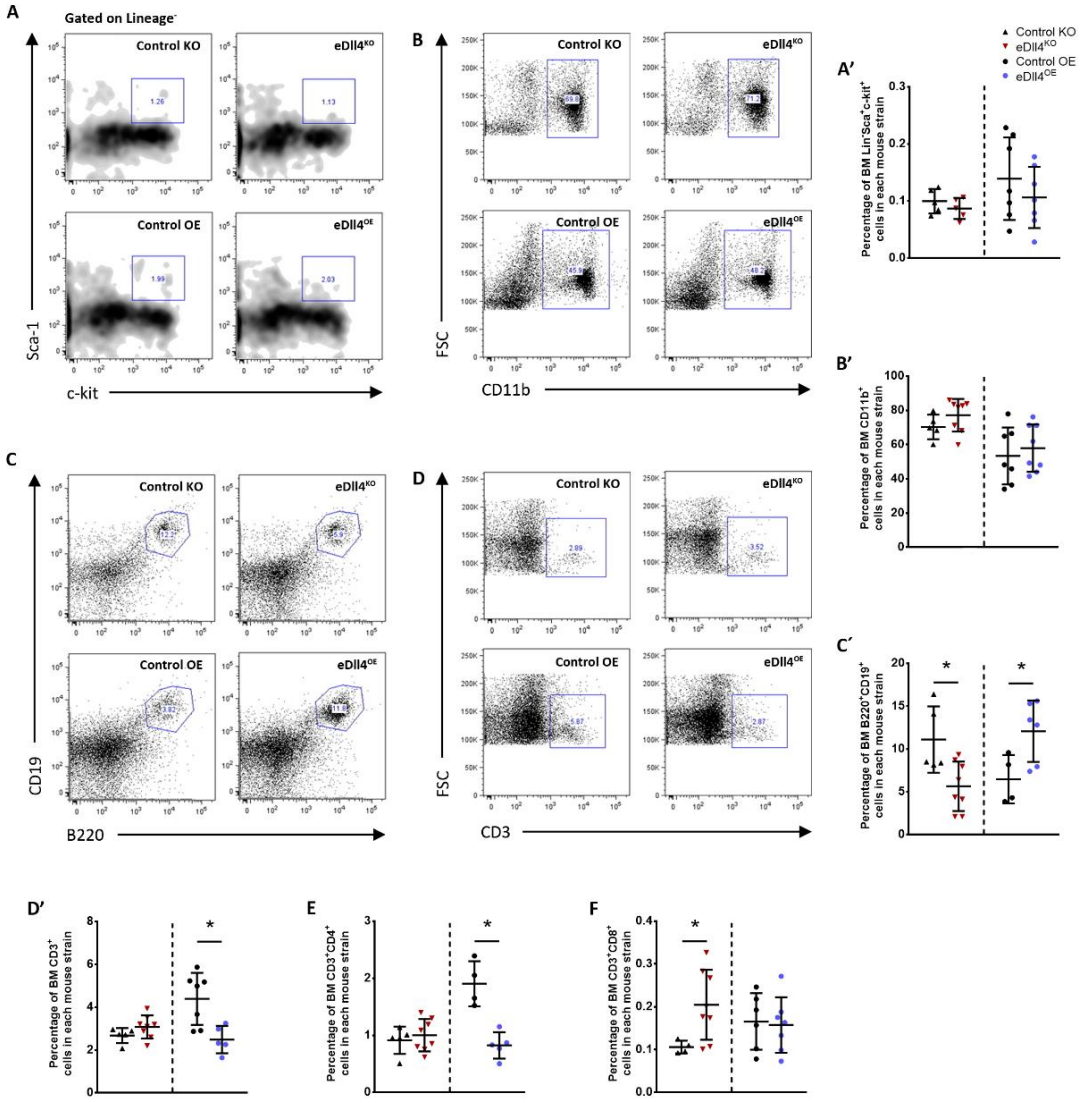

Figure 3.6. Endothelial Dll4 modulates the BM lymphoid content 8 days after irradiation

(A-D) Representative plots of the flow cytometric analysis of bone marrow cells from eDll4^{KO}, eDll4^{OE} and the respective controls by day 8 after irradiation. Data were obtained from samples of at least 5 mice. FSC, forward scatter; SSC, side scatter. Quantification of **(A')** LSK and **(B')** CD11b^{+} cell percentage showed that eDll4 does not significant affect the stem or myeloid populations. Analysis of the percentages of **(C')** B220^{+}CD19^{+} B cells, **(D')** CD3^{+} T cells, **(E)** CD3^{+}CD4^{+} T helper cells and **(F)** CD3^{+}CD8^{+} T cytotoxic cells showed that eDll4^{KO} have a decrease in B lymphopoiesis and an increase in cytotoxic T cells and that eDll4^{OE} favors B cell instead of T commitment, with a particular decrease in T-helper cells. Data are means ± SD of at least 5 mice (* $p<0.05$).

Analysis of our mutant mice 26 days after sub-lethal irradiation showed that modulation of eDll4 also affected BM (**Figure 3.8**) and PB hematopoietic content (**Figure 3.9**) in late stages of recovery. Particularly, knocking out eDll4 induced a decrease in BM myeloid content from 63,08% ± 9,90 in Control KO mice to 47,23% ± 15,04 in eDll4^{KO} mice

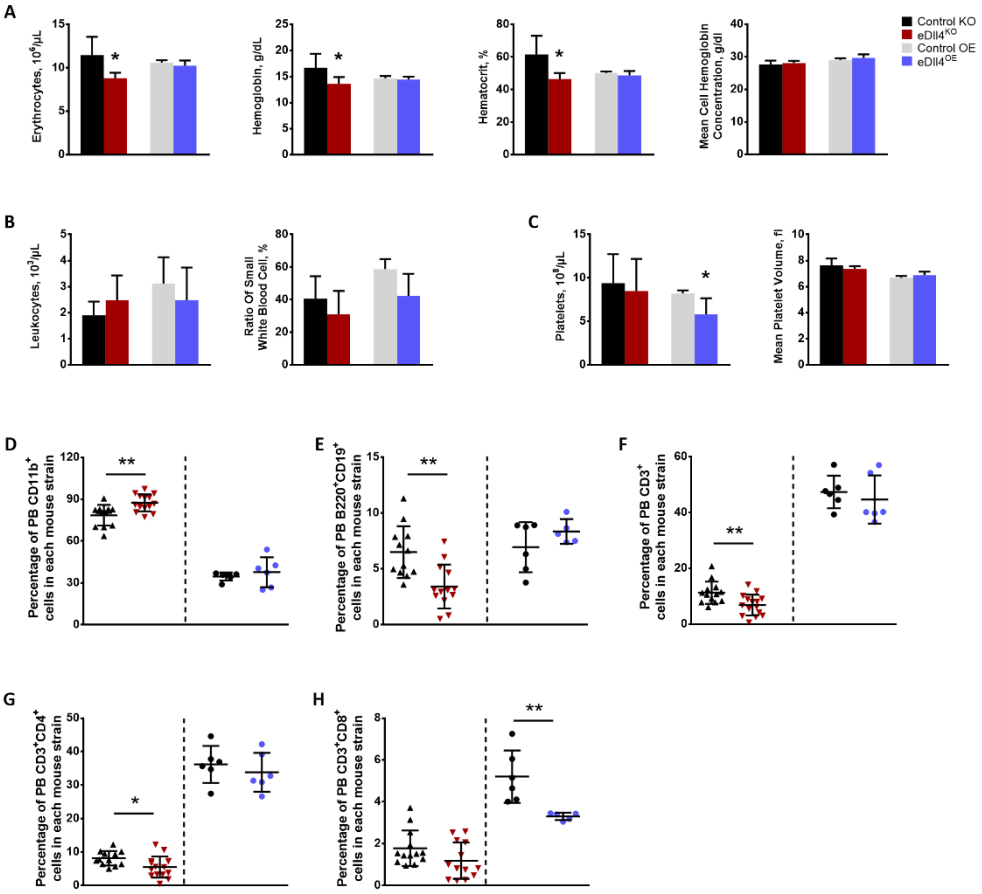

Figure 3.7. eDll4 modulates erythrocyte and platelet levels and the percentage of peripheral blood myeloid and lymphoid cells 8 days after myeloablation

(A) Erythrocyte counts, hemoglobin, hematocrit, and mean cell hemoglobin concentration were assessed by complete blood counts, showing that eDll4 knockout decreases the erythroid lineage. **(B)** Leukocytes and the ratio of small over large white blood cells were also assessed, showing that endothelial Dll4 does not affect leukopoiesis. **(C)** Platelet levels were decreased in eDll4^{OE} mice, but eDll4 did not affect mean platelet number. Data are means ± SD of at least 4 mice (* $p<0.05$). **(D-H)** Flow cytometric analysis of peripheral blood hematopoietic lineages shows that **(D)** CD11b^{+} myeloid cells increased and **(E)** B220^{+}CD19^{+} B, **(F)** CD3^{+} T and **(G)** CD3^{+}CD4^{+} T helper cells decreased in eDll4^{KO} mice, but were not affected in eDll4^{OE} mice. **(H)** CD3^{+}CD8^{+} T cytotoxic cell analysis revealed a significant increase in eDll4^{OE} but not in Dll4^{KO} mice. Data are means ± SD of at least 5 mice (* $p<0.05$, ** $p<0.01$).

(**Figure 3.8A**), without affecting the lymphoid compartment (**Figure 3.8B and C**). Nonetheless, these mice had lower levels of T helper cells (73% of Control KO) (**Figure 3.8D**), but not of cytotoxic T lymphocytes (**Figure 3.8E**), and presented a 2.2 fold increase in HSPCs (Lin^{-}Sca^{+}c-kit^{+}) when compared to the control mice (**Figure 3.8F**). On the other hand, endothelial Dll4 overexpression only caused a reduced recovery of T lymphocytes to about 84% of the levels found in their controls (**Figure 3.8C**).

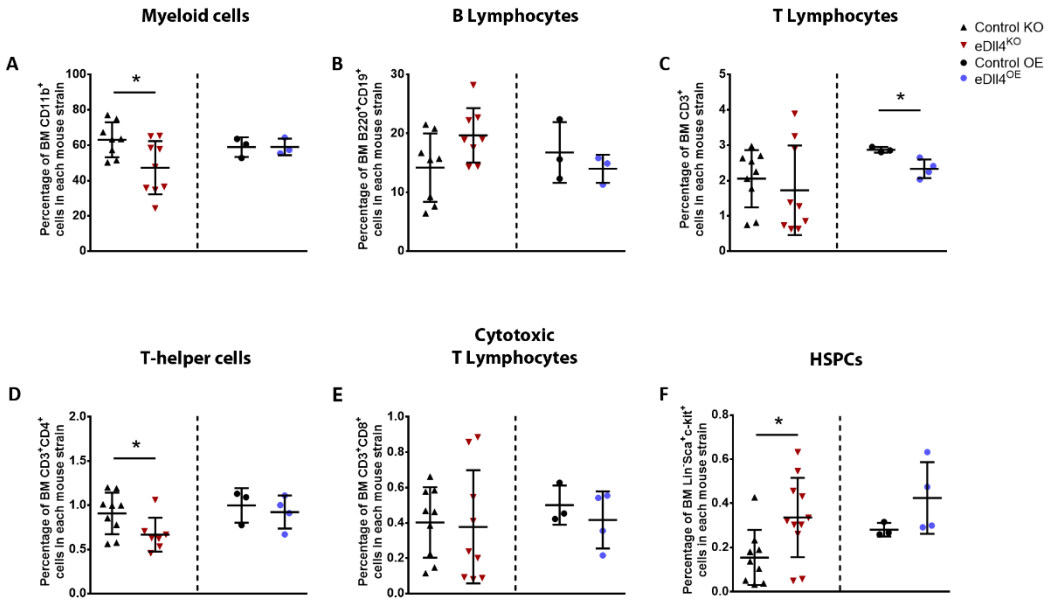

Figure 3.8. Modulation of eDll4 induces differential BM hematopoietic recovery by day 26 post-irradiation

Quantification of BM hematopoietic lineages by flow cytometry shows that 26 days after myeloablation the **(A)** $CD11b^+$ cell percentage is significantly decreased in $eDll4^{KO}$ mice. Regarding the lymphoid compartment, **(B)** $B220^+CD19^+$ B cells were not significantly modified but **(C)** $CD3^+$ T lymphocytes showed lower levels in $eDll4^{OE}$ mice than in the control counterparts. **(D)** $CD3^+CD4^+$ T helper cells were only decreased in $eDll4^{KO}$, but not in $eDll4^{OE}$ mice. **(E)** $CD3^+CD8^+$ T cytotoxic cells were not affected by eDll4 modulation. **(F)** LSK HSPCs were higher in $eDll4^{KO}$ mice but were not significantly affected by eDll4 overexpression. Data are means ± SD of at least 3 mice (* $p<0.05$).

Although both mouse models revealed a similar recovery of the blood parameters (**Figure 3.9A, B and C**), the myeloid and lymphoid composition after 26 days of recovery was markedly affected by eDll4 levels. Specifically, endothelial Dll4 positively correlated with $CD11b^+$ myeloid cells, decreasing upon eDll4 knockout (from 44,08% ± 17,12 in Control KO to 24,78% ± 10,16 in $eDll4^{KO}$ mice) and increasing with eDll4 overexpression (31,80% ± 4,57 and 44,88% ± 5,88 in Control OE and $eDll4^{OE}$ mice, respectively) (**Figure 3.9D**). A similar phenotype was found for circulating B cells, where $eDll4^{KO}$ mice showed a tendency towards a reduction in B cells and $eDll4^{OE}$ mice exhibited a 1.4 fold increase in the percentage of B cells upon 26 days of recovery (**Figure 3.9E**). T cells, on the other hand, negatively correlated with eDll4 levels; whereas $eDll4^{KO}$ mice showed an increase from 18,76% ± 8,02 (Control KO) to 25,53% ± 3,44, $eDll4^{OE}$ T cell percentage decreased from 31,70% ± 2,00 (Control OE) to 22,18% ± 2,3 (**Figure 3.9F**). This translated into the same correlation for both helper and cytotoxic T cells (**Figure 3.9G and H**), although only helper

T cells were significantly decreased in eDll4^{OE} mice and only cytotoxic T cells were significantly increased in eDll4^{KO} mice.

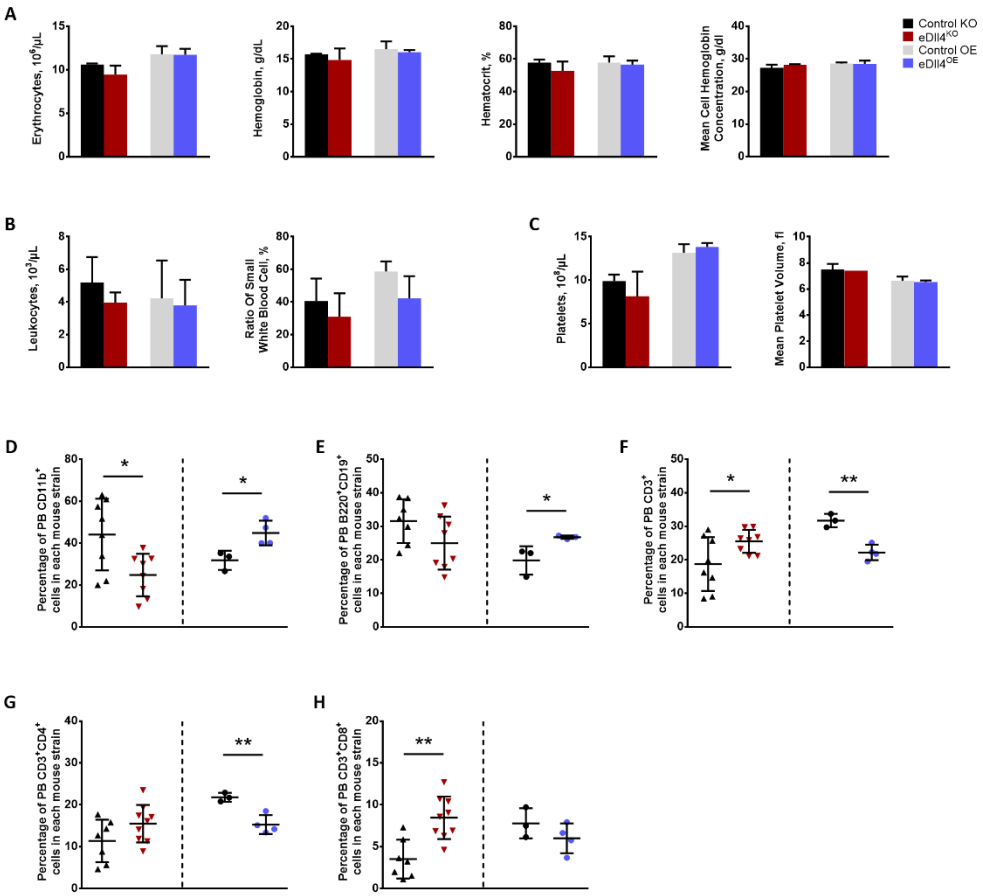

Figure 3.9. eDll4 modulation does not affect complete blood counts but modifies the peripheral blood hematopoietic composition 26 days after irradiation.

(A) Erythrocyte counts, hemoglobin, hematocrit, and mean cell hemoglobin concentration were assessed by complete blood counts showing that eDll4 does not affect erythropoiesis. **(B)** Leukocytes and the ratio of small over large white blood cells were also assessed, revealing that endothelial Dll4 does not affect leukopoiesis. **(C)** Platelet levels and mean platelet levels were also not affected by eDll4 modulation. Data are means ± SD of at least 3 mice (* $p<0.05$). **(D-H)** Flow cytometric analysis of peripheral blood hematopoietic lineages. **(D)** eDll4 levels decrease in eDll4^{KO} mice and increase in eDll4^{OE} mice. **(E)** B220^{+}CD19^{+} B cell content was not affected by eDll4 knockout but increased in eDll4^{OE} mice. **(F)** Total CD3^{+} T lymphocytes were increased in eDll4^{KO} mice and decreased in eDll4^{OE} mice, which translated into **(G)** a decrease in CD3^{+}CD4^{+} T helper cells decreased in eDll4^{OE} mice and **(H)** an increase in CD3^{+}CD8^{+} T cytotoxic cells in eDll4^{KO} mice. Data are means ± SD of at least 3 mice (* $p<0.05$, ** $p<0.01$).

A comparison of sub-lethal irradiated mice by days 8 and 26 of recovery with mice that were not exposed to irradiation (induced and sacrificed on the same time-points as mice from the 26 days post-irradiation group) is shown below. In detail, the BM myeloid content

by day 26 following irradiation severely decreased in eDll4^{KO} mice, but not in Control KO mice, when compared to the 8 day after irradiation time-point. In eDll4^{OE} mice and their controls, however, CD11b^{+} relative percentage remained similar throughout the entire experiment (**Figure 3.10A**). A similar tendency was found for both mice mutants in circulating myeloid cells, although eDll4^{OE} mice recovered to have significantly higher levels of circulating CD11b^{+} cells (**Figure 3.11A**).

Regarding B lymphocytes, eDll4^{KO} mice show a marked decrease in their BM levels at day 8 and by day 26 they increase to levels similar to the ones found in non-irradiated mice. Contrastingly, Control KO mice show similar levels in all time-points. The opposite is found in eDll4^{OE} mice, which reveal similar levels in all time-points, whereas Control OE have decreased B lymphocytes by day 8 and a marked increase by day 26 post-irradiation (**Figure 3.10B**). In both mutants and respective controls, circulating B lymphocytes increase from day 8 to day 26. However, whereas eDll4^{KO} mice always have lower B cell percentage, eDll4^{OE} have higher levels than the control counterparts (**Figure 3.11B**).

Knocking out eDll4 induces a decrease in BM T cell percentage throughout recovery in a more striking manner than in Control KO (**Figure 3.10C**). However, the inverse was detected in PB, where T lymphocytes increased in both eDll4^{KO} and Control KO mice, but more strikingly in eDll4^{KO} mice (**Figure 3.11C**). In eDll4^{OE} mice, BM T cell percentage remained similar throughout recovery, but their levels in circulation decreased. Control OE mice, however, showed an increase in BM levels and a similar decrease in the periphery (**Figure 3.10C and 3.11C**). This suggests that the decrease in circulating T cells in Control OE mice is due to a reduction in their production, but that in eDll4^{OE} mice it may be caused by reduced migration of these cells into the bloodstream.

Helper T cells were found to have similar variations, both in BM and PB, when compared to total CD3^{+} T cells (**Figure 3.10D and 3.11D**). BM CD3^{+}CD8^{+} cytotoxic T cells, however, showed similar levels in both time-points following irradiation, and in all mice, reaching markedly lower levels than the observed in non-irradiated mice (**Figure 3.10E**). Nonetheless, their levels in circulation increased in all mice groups, similarly to what was described for both total and helper T cells in eDll4^{KO} and Control KO mice, but contrary to what was seen in the other T cell subsets in eDll4^{OE} and Control OE mice (**Figure 3.11E**).

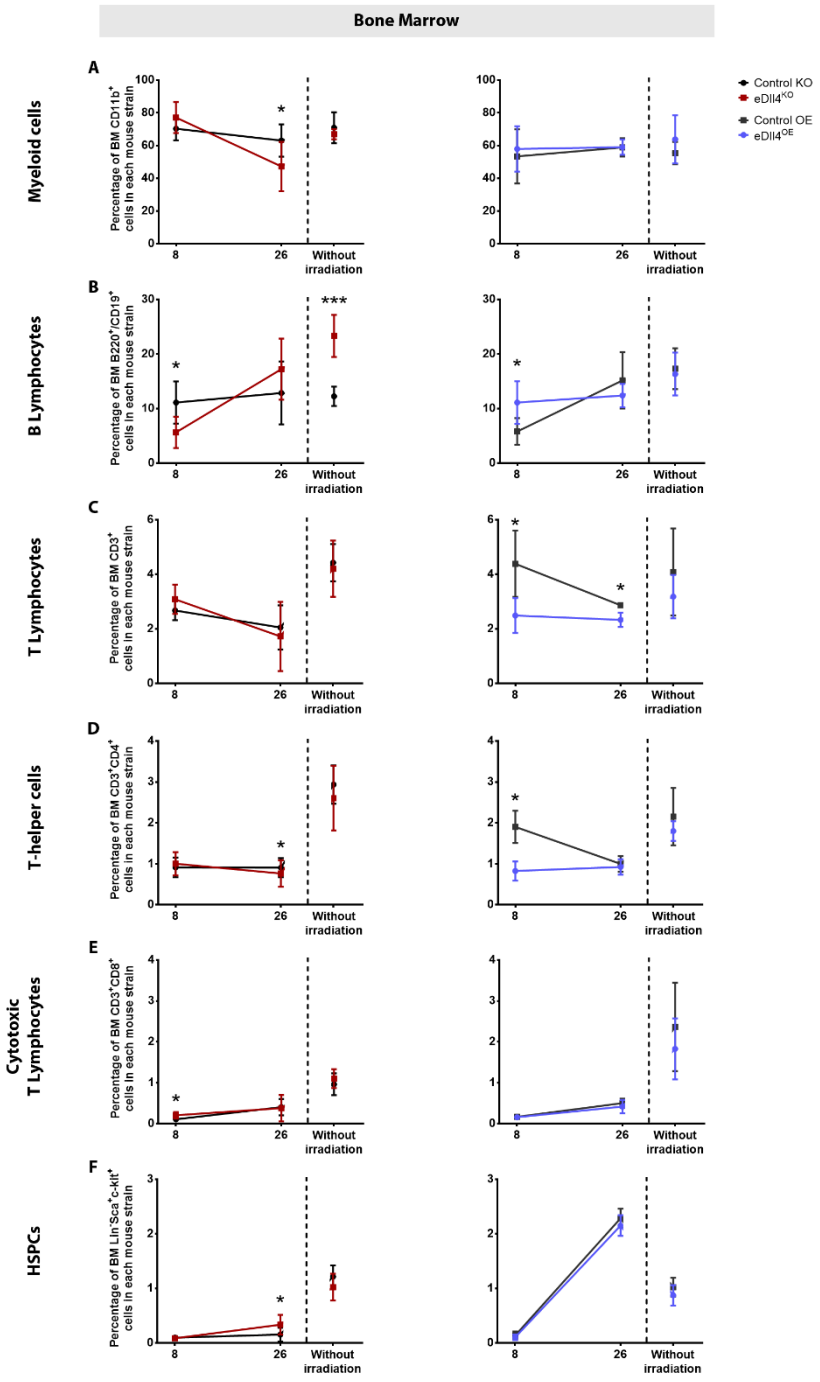

Figure 3.10. Notch ligand Dll4 expression in endothelial cells modulates BM recovery following sub-lethal irradiation

(A) Myeloid cells significantly decrease from day 8 to 26 in eDll4^{KO} mice and not in their controls. However, their levels remain similar throughout recovery in both Control OE and eDll4^{KO} mice. **(B)** In eDll4^{KO} mice, B lymphocyte percentage severely decreases by day 8 after irradiation, but rapidly increases and, by day 26, their levels closely resemble those in non-irradiated mice. In eDll4^{OE} mice, B cell levels remained similar throughout recovery, but in Control OE mice, they severely decreased by day 8 and increased by day 26. **(C)** eDll4^{KO} and Control KO mice had similar T cell levels in all time-points, both decreasing from day 8 to 26 to about half the levels of non-irradiated mice, a behavior that was also identified in Control OE mice, but not in eDll4^{OE} mice, which had similar T cell percentages in all time-points. **(D)** T helper cell levels were similar to the CD3^{+} levels in all time points for both mutant mice and their controls. **(E)** Cytotoxic T cell levels increased from day 8 to 26 in all mice models, but by day 26 they were still lower than the levels found in non-irradiated mice. **(F)** Hematopoietic stem and progenitor cells increased in eDll4^{KO}, but not in Control KO mice, from day 8 to 26 of recovery, but their levels were lower than in non-irradiated mice. Both Control OE and eDll4^{OE} showed a marked increase in HSPC in recovery, to levels higher than in mice without irradiation. Data are means ± SD of at least 3 mice (* $p<0.05$).

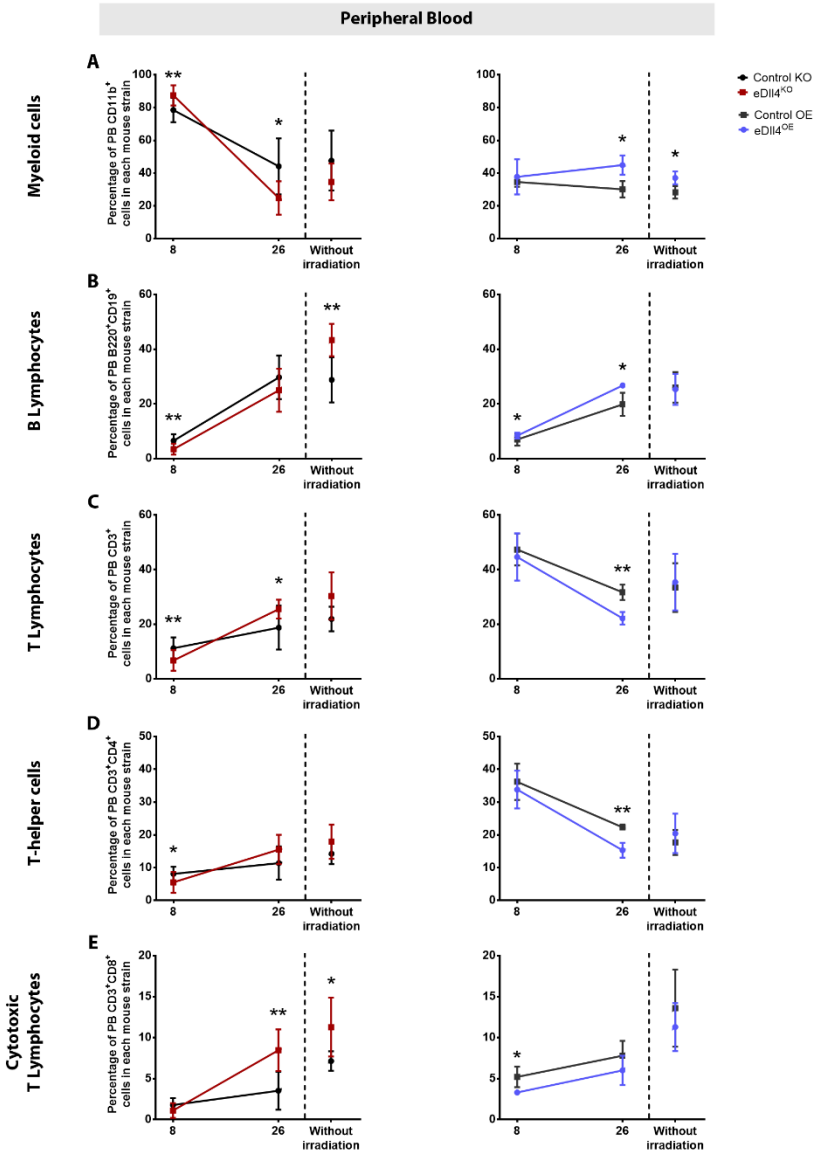

Figure 3.11. The recovery of PB hematopoietic lineages following irradiation is affected by endothelial Dll4

(A) Myeloid cells significantly decrease from day 8 to 26 in eDll4^{KO} and Control KO mice. However, their levels remain similar throughout recovery in both Control OE and eDll4^{KO} mice, with a slight increase in eDll4^{OE} mice. **(B)** Both mice mutants and their controls showed increasing levels of B lymphocytes through recovery. However, eDll4KO mice had lower levels than their controls and eDll4^{OE} mice had higher B cell percentage than Control OE mice. **(C)** eDll4^{KO} and Control KO mice had increasing T cell levels during recovery, with a more striking increase in eDll4KO mice. Control OE and eDll4^{OE} mice both had decreasing T cell percentages, but eDll4^{OE} mice exhibited a more marked decrease. **(D)** T helper cell levels were similar to the CD3^{+} levels in all time points for both mutant mice and their controls. **(E)** Cytotoxic T cell levels increased from day 8 to 26 in all mice models, and their levels were higher in eDll4^{KO} mice and lower in eDll4^{OE} mice, compared to the control counterparts. Data are means ± SD of at least 3 mice (* p<0,05, ** p<0,01).

Concerning BM HSPCs, although eDll4^{KO} mice and Control KO revealed significant lower levels of HSPCs after recovery compared to their non-irradiated counterparts, eDll4^{OE} and their controls exhibited a marked increase in HSPCs by day 26 post-irradiation up to 2 fold the levels found in animals that had not been exposed to irradiation (**Figure 3.10F**), suggesting that the differential responses are strain-specific and not caused by eDll4 modulation.

Together, these data show that eDll4 affects BM recovery after myeloablation, mostly by affecting the percentages of BM and PB hematopoietic lineages. The most striking result we found is that endothelial Dll4 negatively correlates with B lymphocyte recovery in the BM but positively correlates with B cell content in circulation, suggesting that eDll4 may be involved in the migration/retention of these cells in the BM.

3.4.4. Endothelial Dll4 depletion affects BM recovery and B cell content after BM transplantation.

Next, we assessed whether the BM and PB changes induced by sub-lethal irradiation of eDll4^{KO} mice could be reversed through transplantation of total bone marrow from normal (Control KO) mice. For this purpose, we lethally irradiated (900 rad) eDll4^{KO}

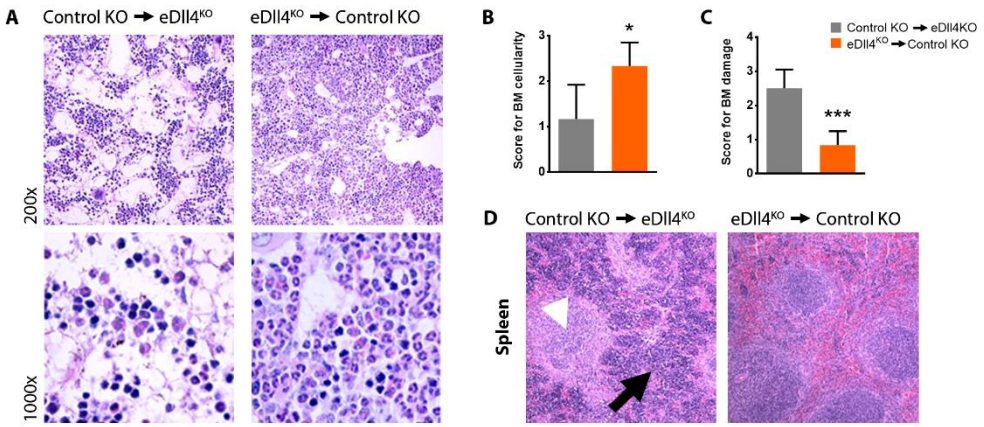

Figure 3.12. Knocking out endothelial Dll4 affects BM recovery after BM transplantation

(A) Hematoxylin and eosin staining of BM sections of eDll4^{KO} and Control KO mice 26 days after BM transplantation with Control KO and eDll4^{KO} BM, respectively showing fibrin deposits associated with loss of well-defined sinusoids in eDll4^{KO} recipient mice. **(B)** BM Cellular density and **(C)** damage were assessed according to a grading score, ranging from 0 (minimal) to 3 (high). **(D)** Hematoxylin and eosin staining of liver sections show hyperplasia of the red pulp (black arrow) and depletion of the white pulp (white arrowhead) in eDll4^{KO} recipient mice. Data are representative of 6 animals per group and scored as mean ± SD (* $p<0.05$, *** $p<0.001$).

recipient mice, which were subsequently transplanted with BM harvested from Control KO mice 26 days after injection with castor oil. Similarly, we also transplanted total BM of eDll4^{KO} mice, harvested 26 days after induction with tamoxifen, into Control KO mice. Twenty-six days after BM transplantation (BMT), eDll4^{KO} mice that had received BM from Control KO mice revealed a higher degree of BM fibrin deposits and damage to the sinusoids and decreased cell density than Control KO mice transplanted with eDll4^{KO} BM, as assessed by hematoxylin and eosin (HE) staining of BM sections (**Figure 3.12A, B and C**). Hyperplasia of the red pulp and lymphoid/white pulp depletion were also detected in the spleen of eDll4^{KO} host mice (**Figure 3.12D**), but no changes were seen in the liver or thymus (data not shown).

Analysis of the BM and PB hematopoietic lineages in these mice revealed that the percentages of myeloid and lymphoid compartments correlate with those obtained for the sub-lethal irradiated donor mice. Particularly, the percentage of CD11b^{+} myeloid cells in the circulation of Control KO mice that received a BM transplant from eDll4^{KO} mice was lower than when the opposite BMT was performed (**Figure 3.13A and A'**). Furthermore, both BM and PB CD19^{+}B220^{+} B cells were higher when eDll4^{KO} mice were used as donors

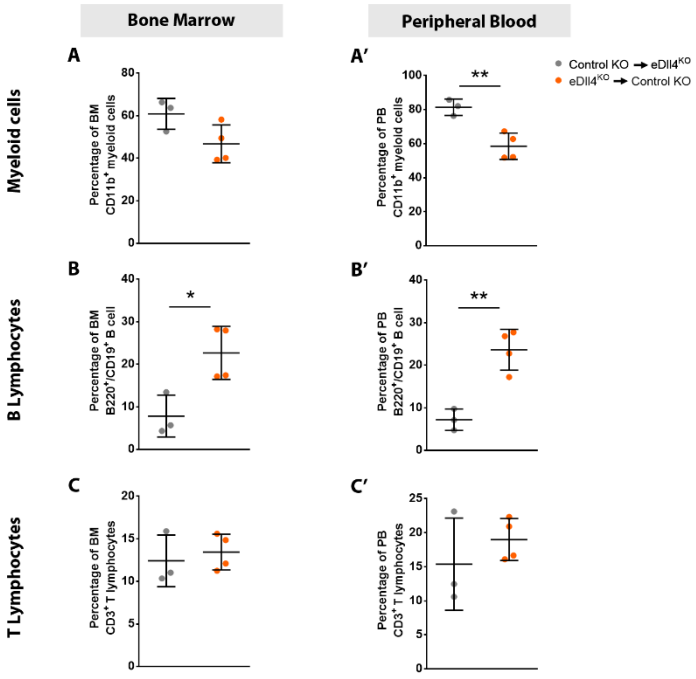

Figure 3.13. Endothelial Dll4 knockout in donor mice modulates myeloid and B lymphoid percentages after BM transplantation

Quantification of BM hematopoietic lineages by flow cytometry shows that 26 days after BM transplantation **(A and A')** CD11b^{+} cell percentage is significantly decreased in Control KO recipient mice, when compared to eDll4^{KO} recipient mice. **(B and B')** The B lymphoid compartment was also assessed, showing that B220^{+}CD19^{+} B cells were significantly higher when eDll4^{KO} mice were used as donors, than when Control KO BM was transplanted into eDll4^{KO} mice. **(C and C')** CD3^{+} T lymphocytes showed no alterations both in the BM and PB compartments. Data are means ± SD of at least 3 mice (* $p<0.05$, ** $p<0.01$).

that when Control KO BM was transplanted into eDll4^{KO} mice (**Figure 3.12B and B´**). T lymphocyte percentage, however, was similar in both groups (**Figure 3.13C and C'**).

Together, these data suggest that knocking out Dll4 in the BM endothelium of donor mice may improve hematopoietic recovery following lethal myeloablation.

3.5. DISCUSSION

In this Chapter, we took advantage of previously established mouse models to specifically modulate the levels of Notch ligand Delta-like 4 in endothelial cells and to study its involvement in hematopoiesis. Dll4 has been extensively linked with hematopoiesis, regulating not only HSC quiescence, self-renewal and differentiation (Lahmar et al. 2008; Dando et al. 2005; Karanu et al. 2001; Lauret et al. 2004), but also the binary cell fate determination of the lymphoid lineages from a common lymphoid progenitor (Koch et al. 2008; Hozumi et al. 2008; Mohtashami et al. 2010; Coste et al. 2013) and the differentiation of the megakaryocytic (Dorsch et al. 2002; Poirault-Chassac et al. 2010) and erythroid lineages (Dando et al. 2005; Laranjeiro et al. 2012). The increasing reports of the role of the BM vascular niche in hematopoietic stem and progenitor cell maintenance (Kunisaki et al. 2013; He et al. 2014; Gori et al. 2015; H.-G. Kopp et al. 2005; Hooper et al. 2009; Kobayashi et al. 2010; Kubota et al. 2008; Nombela-Arrieta et al. 2013) led us to hypothesize that Dll4 modulation in the BM vascular niche could be affecting hematopoiesis.

Our results show that both knocking out Dll4 in VE-Cadherin^{+} cells and overexpressing Dll4 in Tie2^{+} cells in a setting of BM recovery after myeloablation induced qualitative changes in hematopoiesis and affected BM recovery following myeloablation. Recently, Remédio et al. showed that anti-Dll4 treatment of donor BM improved BM hematopoietic recovery following transplantation into lethally irradiated recipients (Remédio et al. 2012). Our data suggests that this was mostly caused by endothelial Dll4 blockade, as we observed that transplantation of eDll4^{KO} total BM into lethally irradiated Control KO mice improved BM recovery, increasing its cellularity and minimizing BM damage. Furthermore, after total body sub-lethal irradiation, we show that BM cellularity is inversely proportional to eDll4 levels, as by day 26 into recovery, mice with lower eDll4 levels (eDll4^{KO} and Control OE)

showed a total BM cell number that resembled their non-irradiated counterparts more closely than Control KO and eDll4^{OE} mice.

Although none of the mutant mice showed particular differences in complete blood counts either at steady state or 26 days after myeloablation, by day 8 post-irradiation, eDll4^{KO} mice exhibited decreased levels of erythrocytes, and eDll4^{OE} mice had lower platelet numbers in circulation, compared to the respective controls. Notch signaling has been widely implicated in erythrocyte and megakaryocyte development. In murine erythroleukemia cells, Notch1 promoted erythroid differentiation by preventing apoptosis in committed progenitors (Shelly et al. 1999) and tamoxifen-induced expression of the Notch1 intracellular domain (ICN1) in a cell line that differentiates into myeloid and erythroid cells resulted into increased differentiation of these cells towards the erythroid lineage (Henning et al. 2007). Moreover, although Notch2 is dispensable at steady state, gain- and loss-of-function models of Notch2 signaling have demonstrated that it is required for recovery following stress-induced depletion of erythroid progenitors (Oh et al. 2013). Consistently, embryonic liver HSCs co-culture with Dll4-overexpressing stroma cells and Dll4$^{-/-}$ embryoid bodies assays demonstrated that Dll4 is necessary for primitive erythropoiesis (Dando et al. 2005; Laranjeiro et al. 2012). Contrastingly, Dll4 strongly impairs platelet production by reducing the generation of mature megakaryocytes (MK), without affecting the earlier steps of MK differentiation (Poirault-Chassac et al. 2010; Yan et al. 2001). Our results suggest that expression of Dll4 by endothelial cells is involved in the regulation of both erythroid and megakaryoid differentiation.

Our data also suggest that distinct types of endothelial cells may regulate different hematopoietic lineages. Whereas Tie2 is more abundantly expressed in arteries, arterioles and transitional capillaries (Li et al. 2009), VE-Cadherin is predominantly expressed in BM sinusoids (Butler, Nolan, et al. 2010; Kunisaki et al. 2013), indicating that arteriolar-derived Dll4 regulates the megakaryoid lineage, while Dll4 expression in sinusoid endothelial cells controls the erythroid lineage differentiation.

Analysis of the relative proportions of the different hematopoietic lineages in the BM and PB suggest that mice with lower levels of endothelial Dll4 (eDll4^{KO} and Control OE) may be more susceptible to hematopoietic changes after irradiation, since they have higher variations in the percentages of myeloid and lymphoid compartments by day 8 post-

irradiation than Control KO and eDll4^{OE} mice. Nonetheless, by day 26 following myeloablation, these mice had reached relative proportions of hematopoietic cells that were more similar to their non-irradiated controls than Control KO and eDll4^{OE} mice, suggesting that endothelial Dll4 may prevent a normal BM reconstitution in later stages.

The discrepancies found in cell percentages at steady state and in the changes caused by myeloablation in both mouse models are most likely because the genetic background of Control KO and eDll4^{KO} mice is different than that of Control OE and eDll4^{OE} mice, which is often a source of variation of relative hematopoietic cell percentages (Chen & Harrison 2002). Consistent with our data, whereas C57/B6J (eDll4^{KO}) mice have high levels of circulating B cells and low levels of CD4^{+} and CD8^{+} T lymphocytes, FVB/NJ (eDll4^{OE}) mice show the inverse proportions of B and T cells, with relative proportions of myeloid cells similar to C57/B6J mice (Petkova et al. 2008).

An extensive collection of data suggested the Notch signaling is critical player in binary cell fate determination of lymphoid lineages. Physiologically, Notch signaling acts at the early lymphoid progenitor stage (Han et al. 2002), instructing the differentiation along the T cell lineage and functioning as an inhibitory signal for maturation of B cell lineages (Hozumi et al. 2003; Jaleco et al. 2001; Pui et al. 1999; Han et al. 2002; Wilson et al. 2001). Dll4 seems to be crucial in this regulatory process as culture of HSPCs with Dll4-overexpressing stroma cells induces T-cell differentiation and impairs B cell differentiation (Mohtashami et al. 2010). *In vivo*, Dll4 depletion in thymic epithelial cells (*Foxn1-Cre/Dll4$^{lox/lox}$*) also blocks T cell development at the most immature stage of thymocytes and induces the ectopic appearance of immature B cells in the thymus (Koch et al. 2008; Hozumi et al. 2008). Furthermore, upregulation of Dll4 in erythroblasts revealed that Dll4-Notch1 signaling must be maintained at low levels in the BM to prevent premature differentiation of HSCs into T cells (Lee et al. 2013). We show for the first time that endothelial-derived Dll4 is also involved in the B versus T-lymphoid lineage fate determination, suggesting that Dll4-Notch signaling derived modulation of the lymphoid lineage is independent of the microenvironment. It is however unclear if this modulation is occurring by BM endothelial-induced HSC differentiation or whether the thymic endothelium is affecting CLP commitment, or both.

Lethal irradiation causes an acute injury to the bone marrow, suppressing hematopoiesis and significantly damaging the endothelium in the BM sinusoids (H. G. Kopp et al. 2005; Hooper et al. 2009; Li et al. 2008; Li et al. 2010). BM sinusoid repair is a critical step to restore normal hematopoiesis after lethal-irradiation (H. G. Kopp et al. 2005; Avecilla et al. 2004; Hooper et al. 2009; Salter et al. 2009). Although endothelial cell transplantation alone was described to protect lethally irradiated hosts in a similar fashion as total BM transplantation (Chute et al. 2007; Li et al. 2010; Hooper et al. 2009; Salter et al. 2009), BM sinusoids remain predominantly host-derived following BM transplantation (Slayton et al. 2007; Li et al. 2010; Chute et al. 2007) due to DNA repair mechanisms and proliferation of host endothelial cells within the sinusoids that maintain the structural integrity of the BM vascular niche (Li et al. 2008).

We have shown that transplantation of BM from mice lacking endothelial Dll4 into Control KO mice - but not Control KO BM transplantation into lethally irradiated eDll4^{KO} mice - results in an increase in B cell differentiation similar to the one found in sub-lethally irradiated eDll4^{KO} mice. Since both the BM and thymic endothelium are host derived, we hypothesize that BM cells harvested from eDll4^{KO} mice are already committed into the B lymphoid lineage. Analyzing mice in a later time-point to evaluate whether the host derived endothelial cells reverted this commitment in a late stage of recovery following BMT would be required.

It is also interesting to note that after recovery from sub-lethal irradiation (26d), eDll4 expression correlates with low levels of BM B lymphocytes and high levels of circulating B cells. This suggests that endothelial cells with low levels of Dll4 tend to retain B cells in the BM and decrease their mobilization into circulation whereas endothelial cells with high levels of Dll4 increase the mobilization of B cells into the periphery with consequent decrease of the BM relative proportions of these cells.

Taken together, our results show that endothelial Dll4 may hamper BM recovery after myeloablation and simultaneously affect the relative proportions of the hematopoietic lineages, promoting T cell in detriment of B cell differentiation, decreasing platelet production and increasing the erythroid lineage, through mechanisms that are most likely differentially regulated by distinct endothelial subsets.

3.6. REFERENCES

Avecilla, S.T. et al., 2004. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nature medicine*, 10(1), pp.64–71.

Banna, G.L. et al., 2004. Nonmyeloablative allogeneic stem cell transplantation (NST) after truly nonmyeloablative and reduced intensity conditioning regimens. *Critical Reviews in Oncology/Hematology*, 51(3), pp.171–189.

Boulais, P.E. & Frenette, P.S., 2015. Making sense of hematopoietic stem cell niches. *Blood*, 125(17), pp.2621–9.

Bradley, T.R. & Metcalf, D., 1966. The growth of mouse bone marrow cells in vitro. *The Australian journal of experimental biology and medical science*, 44(3), pp.287–99.

Butler, J.M., Nolan, D.J., et al., 2010. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell*, 6(3), pp.251–264.

Butler, J.M., Kobayashi, H., et al., 2010. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nature reviews. Cancer*, 10(2), pp.138–146.

Calvi, L.M. et al., 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*, 425(6960), pp.841–846.

Lo Celso, C. & Scadden, D.T., 2011. The haematopoietic stem cell niche at a glance. *Journal of Cell Science*, 124(21), pp.3529–3535.

Champlin, R. et al., 2000. Harnessing graft-versus-malignancy: Non-myeloablative preparative regimens for allogeneic haematopoietic transplantation, an evolving strategy for adoptive immunotherapy. *British Journal of Haematology*, 111(1), pp.18–29.

Chen, J. & Harrison, D.E., 2002. Quantitative trait loci regulating relative lymphocyte proportions in mouse peripheral blood Quantitative trait loci regulating relative lymphocyte proportions in mouse peripheral blood. , 99(2), pp.561–566.

Chute, J.P. et al., 2007. Transplantation of vascular endothelial cells mediates the hematopoietic recovery and survival of lethally irradiated mice. *Blood*, 109(6), pp.2365–72.

Coste, A.D. La et al., 2013. In Vivo and in Absence of a Thymus, the Enforced Expression of the Notch Ligands Delta-1 or Delta-4 Promotes T Cell Development with Specific Unique Effects. *The Journal of Immunology*.

Coulombel, L., 2004. Identification of hematopoietic stem/progenitor cells: strength and drawbacks of functional assays. *Oncogene*, 23(43), pp.7210–7222.

Dando, J.S. et al., 2005. Notch/Delta4 interaction in human embryonic liver CD34+ CD38- cells: positive influence on BFU-E production and LTC-IC potential maintenance. *Stem cells*, 23(4), pp.550–560.

Ding, L. et al., 2012. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*, 481(7382), pp.457–62.

Ding, L. & Morrison, S.J., 2013. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature*, 495(7440), pp.231–5.

Doan, P.L. et al., 2013. Tie2(+) bone marrow endothelial cells regulate hematopoietic stem cell regeneration following radiation injury. *Stem cells (Dayton, Ohio)*, 31(2), pp.327–37.

Dorsch, M. et al., 2002. Ectopic expression of Delta4 impairs hematopoietic development and leads to lymphoproliferative disease. *Blood*, 100(6), pp.2046–55.

Duncan, A.W. et al., 2005. Integration of Notch and Wnt signaling in hematopoietic stem cell maintenance.

Nature immunology, 6(3), pp.314–22.

Gori, J.L. et al., 2015. Vascular niche promotes hematopoietic multipotent progenitor formation from pluripotent stem cells. *Journal of Clinical Investigation*, 125(3), pp.1–12.

Hamada, B.T. et al., 1998. Transendothelial Migration of Megakaryocytes in Response to Stromal Cell-derived Factor 1 (SDF-1) Enhances Platelet Formation. , 188(3).

Han, H. et al., 2002. Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *International immunology*, 14(6), pp.637–645.

Hashimoto, M. et al., 2008. S Phase-preferential Cre-recombination in mammalian cells revealed by HIV-TAT-PTD-mediated protein transduction. *Journal of biochemistry*, 143(1), pp.87–95.

He, N. et al., 2014. Bone marrow vascular niche: home for hematopoietic stem cells. *Bone marrow research*, 2014, p.128436.

Henning, K. et al., 2007. mNotch1 signaling and erythropoietin cooperate in erythroid differentiation of multipotent progenitor cells and upregulate ??-globin. *Experimental Hematology*, 35(9), pp.1321–1332.

Himburg, H.A. et al., 2012. Pleiotrophin regulates the retention and self-renewal of hematopoietic stem cells in the bone marrow vascular niche Heather. *Cell Reports*, 2(4), pp.946–975.

Hooper, A.T. et al., 2009. Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2 mediated regeneration of sinusoidal endothelial cells. *Cell stem cell*, 4(3), pp.263–74.

Hozumi, K. et al., 2003. Active form of Notch members can enforce T lymphopoiesis on lymphoid progenitors in the monolayer culture specific for B cell development. *Journal of immunology (Baltimore, Md : 1950)*, 170(10), pp.4973–4979.

Hozumi, K. et al., 2008. Delta-like 4 is indispensable in thymic environment specific for T cell development. *The Journal of experimental medicine*, 205(11), pp.2507–2513.

Jaleco, A.C. et al., 2001. Differential Effects of Notch Ligands Delta-1 and Jagged-1 in Human Lymphoid Differentiation. *J Exp Med*, 194(7), pp.991–1001.

Karanu, F.N. et al., 2001. Human homologues of Delta-1 and Delta-4 function as mitogenic regulators of primitive human hematopoietic cells. *Blood*, 97(7), pp.1960–1967.

Karanu, F.N. et al., 2000. The notch ligand jagged-1 represents a novel growth factor of human hematopoietic stem cells. *The Journal of experimental medicine*, 192(9), pp.1365–1372.

Kiel, M.J. et al., 2009. Hematopoietic Stem Cells Do Not Depend on N-Cadherin to Regulate Their Maintenance. *Cell Stem Cell*, 4(2), pp.170–179.

Kiel, M.J. et al., 2005. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*, 121(7), pp.1109–21.

Kiel, M.J., Radice, G.L. & Morrison, S.J., 2007. Lack of Evidence that Hematopoietic Stem Cells Depend on N-Cadherin-Mediated Adhesion to Osteoblasts for Their Maintenance. *Cell Stem Cell*, 1(2), pp.204–217.

Kimura, Y. et al., 2011. c-Kit-Mediated Functional Positioning of Stem Cells to Their Niches Is Essential for Maintenance and Regeneration of Adult Hematopoiesis. *PLoS One*, 6(10).

Kobayashi, H. et al., 2010. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. , 12(11), pp.1046–1056.

Koch, U. et al., 2008. Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *The Journal of experimental medicine*, 205(11), pp.2515–2523.

Koch, U. & Radtke, F., 2011. Notch in T-ALL: new players in a complex disease. *Trends in immunology*, 32(9), pp.434–42.

Kopp, H.G. et al., 2005. Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. *Blood*, 106(2), pp.505–513.

Kopp, H.-G. et al., 2005. The Bone Marrow Vascular Niche: Home of HSC Differentiation and Mobilization. *Physiology*, 20, pp.349–356.

Kubota, Y., Takubo, K. & Suda, T., 2008. Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche. *Biochemical and Biophysical Research Communications*, 366, pp.335–339.

Kunisaki, Y. et al., 2013. Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature*, 502(7473), pp.637–643.

Lahmar, M. et al., 2008. Distinct effects of the soluble versus membrane-bound forms of the notch ligand delta-4 on human CD34+CD38low cell expansion and differentiation. *Stem cells (Dayton, Ohio)*, 26(3), pp.621–9.

Laranjeiro, R. et al., 2012. The notch ligand delta-like 4 regulates multiple stages of early hemato-vascular development. *PLoS ONE*, 7(4).

Lauret, E. et al., 2004. Membrane-bound delta-4 notch ligand reduces the proliferative activity of primitive human hematopoietic CD34+CD38low cells while maintaining their LTC-IC potential. *Leukemia*, 18(4), pp.788–97.

Lee, S.U. et al., 2013. LRF-mediated Dll4 repression in erythroblasts is necessary for hematopoietic stem cell maintenance. *Blood*, 121(6), pp.918–929.

Li, B. et al., 2010. Endothelial cells mediate the regeneration of hematopoietic stem cells. *Stem Cell Research*, 4(1), pp.17–24.

Li, X.M. et al., 2008. Bone marrow sinusoidal endothelial cells undergo nonapoptotic cell death and are replaced by proliferating sinusoidal cells in situ to maintain the vascular niche following lethal irradiation. *Experimental Hematology*, 36(9), pp.1143–1156.

Li, X.M. et al., 2009. High levels of acetylated low-density lipoprotein uptake and low tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2 (Tie2) promoter activity distinguish sinusoids from other vessel types in murine bone marrow. *Circulation*, 120(19), pp.1910–1918.

Liu, J. et al., 2010. *Notch signaling in the regulation of stem cell self-renewal and differentiation*, Elsevier Inc.

Mohtashami, M. et al., 2010. Direct comparison of Dll1- and Dll4-mediated Notch activation levels shows differential lymphomyeloid lineage commitment outcomes. *Journal of immunology (Baltimore, Md. : 1950)*, 185(2), pp.867–876.

Monvoisin, A. et al., 2006. VE-cadherin-CreER ^{T2} transgenic mouse: A model for inducible recombination in the endothelium. *Developmental Dynamics*, 235(12), pp.3413–3422.

Morrison, S.J. & Scadden, D.T., 2014. The bone marrow niche for haematopoietic stem cells. *Nature*, 505(7483), pp.327–34.

Nombela-Arrieta, C. et al., 2013. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nature cell biology*, 15(5), pp.533–43.

Oh, P. et al., 2013. In vivo mapping of notch pathway activity in normal and stress hematopoiesis. *Cell Stem Cell*, 13(2), pp.190–204.

Orkin, S.H., 2000. Diversification of haematopoietic stem cells to specific lineages. *Nature reviews. Genetics*, 1(1), pp.57–64.

Petkova, S.B. et al., 2008. Genetic influence on immune phenotype revealed strain-specific variations in peripheral blood lineages. *Physiological genomics*, 34(3), pp.304–314.

Poirault-Chassac, S. et al., 2010. Notch/Delta4 signaling inhibits human megakaryocytic terminal

differentiation. *Blood*, 116(25), pp.5670–5678.

Pui, J.C. et al., 1999. Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity*, 11, pp.299–308.

Purton, L.E. & Scadden, D.T., 2012. The hematopoietic stem cell niche. *Frontiers in bioscience (Landmark edition)*, 17, pp.30–9.

Radtke, F., Fasnacht, N. & MacDonald, H.R., 2010. Notch Signaling in the Immune System. *Immunity*, 32(1), pp.14–27.

Radtke, F., MacDonald, H.R. & Tacchini-Cottier, F., 2013. Regulation of innate and adaptive immunity by Notch. *Nature reviews. Immunology*, 13(6), pp.427–37.

Rafii, S. et al., 1995. Human bone marrow microvascular endothelial cells support long-term proliferation and differentiation of myeloid and megakaryocytic progenitors. *Blood*, 86(9), pp.3353–3363.

Remédio, L. et al., 2012. Context- and cell-dependent effects of Delta-like 4 targeting in the bone marrow microenvironment. *PloS one*, 7(12), p.e52450.

Salter, A.B. et al., 2009. Endothelial progenitor cell infusion induces hematopoietic stem cell reconstitution in vivo. *Blood*, 113(9), pp.2104–2107.

Schofield, R., 1978. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood cells*, 4(1–2), pp.7–25.

Shelly, L.L., Fuchs, C. & Miele, L., 1999. Notch-1 inhibits apoptosis in murine erythroleukemia cells and is necessary for differentiation induced by hybrid polar compounds. *J Cell Biochem*, 73(2), pp.164–175.

Sipkins, D.A. et al., 2005. In vivo imaging of specialized bone marrow endothelial microdomains for tumor engraftment. *Nature*, 435(7044), pp.969–973.

Slayton, W.B. et al., 2007. The role of the donor in the repair of the marrow vascular niche following hematopoietic stem cell transplant. *Stem cells (Dayton, Ohio)*, 25(11), pp.2945–55.

Stier, S. et al., 2002. Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. *Blood*, 99(7), pp.2369–2378.

Sugiyama, T. & Nagasawa, T., 2012. Bone marrow niches for hematopoietic stem cells and immune cells. *Inflammation & allergy drug targets*, 11(3), pp.201–6.

Trindade, A. et al., 2012. Low-dosage inhibition of Dll4 signaling promotes wound healing by inducing functional neo-angiogenesis. *PloS one*, 7(1), p.e29863.

Trindade, A. et al., 2008. Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos. *Blood*, 112(5), pp.1720–9.

Varnum-Finney, B. et al., 1998. The Notch ligand, Jagged-1, influences the development of primitive hematopoietic precursor cells. *Blood*, 91(11), pp.4084–4091.

Wilson, A., MacDonald, H.R. & Radtke, F., 2001. Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *The Journal of experimental medicine*, 194(7), pp.1003–12.

Winkler, I.G. et al., 2012. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nature Medicine*, 18(11), pp.1651–1657.

Yan, X.Q. et al., 2001. A novel notch ligand, Dll4, induces T-cell leukemia/lymphoma when overexpressed in mice by retroviral-mediated gene transfer. *Blood*, 98(13), pp.3793–3799.

4.

ENDOTHELIAL DELTA-LIKE 4 IS REQUIRED FOR NORMAL BONE MARROW VASCULAR NICHE RECOVERY AND HEMATOPOIETIC CELL MIGRATION FOLLOWING MYELOABLATION

In preparation. Alvarez-Martins I., Duarte A., Dias S.

CONTENTS

4.1. ABSTRACT

Delta-like 4 (Dll4) is a transmembrane ligand of the Notch signaling pathway which is expressed in arterial blood vessels, sprouting endothelial cells and bone marrow (BM) sinusoids. Dll4 is not only essential for normal vascular development, it is also involved in hematopoiesis. Consistently, following an injury to the BM, endothelial-specific Dll4 decreases B cell content and hampers BM hematopoietic recovery. In this Chapter, we sought to understand the role of endothelial Dll4 in the recovery of BM sinusoids following myeloablation and whether it affected hematopoietic cells through modulation of angiocrine gene expression. We used endothelial-specific Dll4 loss- and gain-of function mouse models (eDll4^{KO} and eDll4^{OE}) and analyzed them at steady state and 8 and 26 days after myeloablation induced by sub-lethal irradiation. No differences were found in vessel number or identity at steady state or 26 days after irradiation in any of the mutant mice but, by day 8 post-irradiation, eDll4^{KO} had an increased number of VE-Cadherin^{+} vessels, and endothelial Dll4 (eDll4) levels negatively correlated with VEGFR2-positive vessel number. The localization of B cells and megakaryocytes (MKs) within the vascular niche also shifted 8 days after myeloablation. The percentage of B cells in vicinity of VE-Cadherin^{+} vessels showed a positive correlation with the levels of eDll4, decreasing in eDll4^{KO} mice and increasing in eDll4^{OE} mice. The percentage of MKs in close contact to VE-Cadherin^{+} vessels showed a similar behavior, but only in eDll4^{KO} mice. *In vitro* migration assays showed that CD34^{+} HSPC migration towards anti-Dll4 treated primary endothelial cells (HUVECs) was decreased, consistent with the downregulation of *Cxcl12* expression in HUVECs. This gene was also downregulated in eDll4^{KO} mice suggesting that SDF1 is required for B cell and MK normal localization within the BM vascular niche. In eDll4^{OE} mice, however, *Cxcl12* expression was also found to be decreased, suggesting that other factors or other cells from the BM microenvironment may be involved in the recruitment of B cells to the vicinity of BM sinusoids. Together, our data demonstrate that eDll4 modulation does not affect vessel number throughout recovery, but affects vessel identity and angiocrine gene expression, which modulates the migration of hematopoietic cells towards the vessel wall and eventually to the periphery.

4.2. INTRODUCTION

Endothelial cells play critical roles in a multitude of physiological and pathological processes (Cines et al. 1998). In addition to regulating vessel morphology and vasomotor tone, they also modulate tumor growth and inflammatory responses and are involved in the regeneration of tissues, such as the liver and the lung, through the expression of unique repertoires of trophic factors, known as angiocrine factors (Pober & Sessa 2007; Ding et al. 2011; Ding et al. 2010; Hu et al. 2014; Ding et al. 2014; Butler, Nolan, et al. 2010).

In the bone marrow, endothelial cells comprising the BM vascular niche not only provide a cellular platform for the differentiation of lineage-committed progenitors, such as megakaryocyte progenitors (Avecilla et al. 2004), but also express different subsets of angiocrine genes that regulate HSC regeneration and differentiation following acute injuries to the BM, to restore hematopoiesis. (Kobayashi et al. 2010; Ding et al. 2012; Butler, Nolan, et al. 2010; Doan et al. 2013). The Notch signaling pathway seems to be involved in this endothelial regulation as expression of the Notch ligands Jag1 and Jag2 by endothelial cells prevents HSC exhaustion and promotes their self-renewal (Butler, Nolan, et al. 2010; Poulos et al. 2013).

The Notch pathway is an evolutionarily conserved pathway that plays a critical role in vascular development and postnatal angiogenesis, participating in a series of cell fate specification events (Shawber & Kitajewski 2004). Delta-like 4 (Dll4) is a ligand for Notch receptors that is mainly expressed in endothelial cells, in particular arteries and capillaries (Villa et al. 2001; Claxton & Fruttiger 2004; Benedito & Duarte 2005; Shutter 2000), but it can also be detected in BM sinusoidal endothelial cells (Butler, Nolan, et al. 2010; Lee et al. 2013). Deletion of a single *Dll4* allele in mice results in early embryonic lethality [from embryonic day (E)9.5] due to abnormal artery development and disrupted branching morphogenesis (Gale et al. 2004; Duarte et al. 2004; Krebs et al. 2004), consistent with Dll4 role in arteriovenous specification (Duarte et al. 2004) and tip cell selection during sprouting angiogenesis (Hellström et al. 2007; Suchting et al. 2007). In **Chapter 3**, we have shown for the first time that endothelial-specific Dll4 affects different hematopoietic lineages and hampers BM recovery following myeloablation. However, its role in the maintenance of the structurally and functionally unique capillary network formed by sinusoidal endothelial cells has not been studied and is the focus of this Chapter.

We demonstrate for the first time how endothelial Dll4 modulation affects the BM vascular niche following irradiation, focusing particularly on vessel identity and expression of angiocrine factors. Using conditional Dll4 knockdown and overexpression mouse models, we show that loss of endothelial Dll4 following myelosuppression changes vessel identity but not vessel number in the earlier stages of recovery, which is accompanied by a decreased percentage of B cells and megakaryocytes in contact with BM sinusoids, possibly through downregulation of *Cxcl12* gene expression. On the other hand, endothelial Dll4 overexpression increased the percentage of B lymphocytes in the vicinity of BM sinusoidal endothelial cells, but *Cxcl12* was also downregulated, suggesting that other factors may be involved in this regulation.

4.3. METHODS

4.3.1. Animal genotyping

Mice genotyping was carried out using tail snips or ear punch biopsies digested at 55ºC, overnight with constant shaking, in a solution of Laird's buffer (1M Tris HCl pH 8.5, 0,5M EDTA, 20% SDS, 5M NaCl) containing 100µg/mL Proteinase K (Sigma). Following digestion, hair and debris were discarded by spinning tubes at 13.000 rpm for 5 minutes. The supernatant was collected into a fresh tube and DNA was precipitated by adding an equal volume of isopropanol. With a clean, sterile, micropipette tip, the long thread-like precipitate of DNA was transferred into a new tube and dissolved in ddH_2O.

Mice were genotyped using 4µL of DNA solution in a mix containing 250µM dNTPs, 0,35µM of forward and reverse primers (**Table 4.I**), 1U Taq Polimerase (Invitrogen) and 5mM $MgCl_2$ when primers for $Dll4^{lox/lox}$ were used or 2,5mM $MgCl_2$ for the VE-Cadherin-Cre-ER^{T2}, Dll4-Tet-O7 and Tie2-rtTA primer pairs. The PCR program was similar for all primers pairs, and 60ºC was found to the optimal annealing temperature. Expected product sizes are depicted in **Table 4.I**.

Table 4.I. Primers used for genotyping.

Genotype	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size
$Dll4^{lox/lox}$	GTGCTGGGACTGTAGCCACT	TGTTAGGGATGTCGCTCTCC	455 bp
VE-Cadherin-Cre-ER^{T2}	CCAGCTAAACATGCTTCATC	CGCTCGACCAGTTTAGTTAC	350 bp
Dll4-Tet-O7	ATCCACGCTGTTTTGACCTC	GTGGAGACATTGCCAAAGGT	500 bp
Tie2-rtTA	AAGTCATTCCGCTGTGCTCT	GTCTCAGAAGTGGGGGCATA	200 bp

4.3.2. Animal experiments

All experimental procedures performed in this Chapter were previously approved by the Instituto de Medicina Molecular Ethics Committee and executed in strict compliance to the guidelines of the Federation of European Laboratory Animal Science Associations (FELASA). The generation of both $Dll4^{lox/lox}$*VE-Cadherin-Cre-ERT2 ($eDll4^{KO}$) and of Dll4-Tet-O7*Tie2-rtTA ($eDll4^{OE}$) has been previously described (Trindade et al. 2012; Trindade et al. 2008). Briefly, to obtain the loss-of-function mice, $Dll4^{lox/lox}$ mice (in which the coding region for the first three exons of Dll4 is flanked by loxP sites) (Koch & Radtke 2011) were crossed VE-Cadherin-CRE-ERT2 mice (Monvoisin et al. 2006), generating a conditional knockout mice ($eDll4^{KO}$; genetic background of C57/B6J mice). To deplete Dll4 specifically in endothelial cells, mice were induced with tamoxifen IP injection (50 mg/kg/day in castor oil, Sigma) for 5 consecutive days, starting 3 days after sub-lethal irradiation (300 rad) (see **Figure 4.1A** for the detailed experimental setup). Since Cre recombination occurs preferentially in S-phase (Hashimoto et al. 2008), we decided to induce the animals after irradiation to make sure that the endothelial cells were dividing (cycling) at the time of induction. Control mice (Control KO) had the same genotype but were injected with castor oil alone.

The gain-of-function Dll4 mutants were obtained by crossing Tet-O7-Dll4 mice with heterozygous Tie2-rtTA mutant mice (Trindade et al. 2008), generating a conditional Dll4 overexpressing mouse mutant ($eDll4^{OE}$; genetic background of FVB/NJ mice). To induce the Dll4 overexpression under the control of the Tie2 promotor, $eDll4^{OE}$ mice were induced through the administration of 2 mg/mL doxycycline (Sigma) in drinking water, with 2.5% sucrose, starting 7 days before irradiation and throughout the entire experiments (**Figure 4.1B**). Control mice (Control OE) had the same gain-of-function genotype, but were given only sucrose in their drinking water. All mice models - $eDll4^{KO}$, $eDll4^{OE}$ and respective

controls - were sacrificed at 8 and 26 days after sub-lethal irradiation. Non-irradiated mice were sacrificed at the same time-point as those sacrificed 26 days following irradiation, regarding the time of induction.

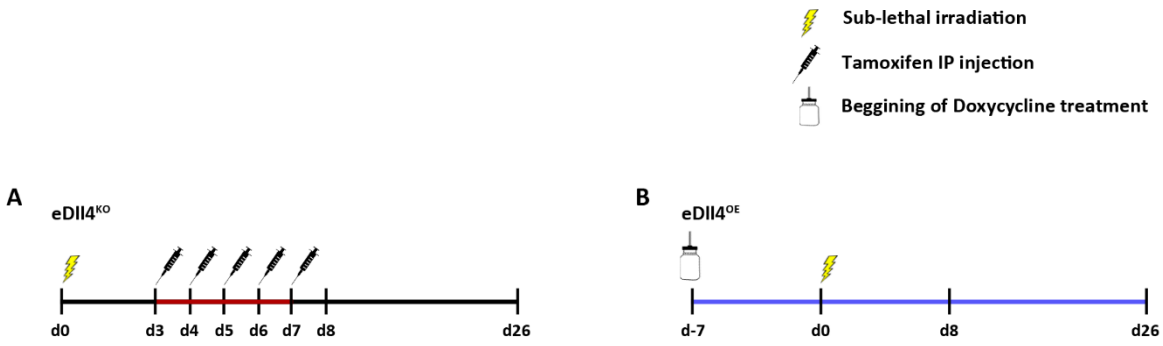

Figure 4.1. Experimental setup

(A) eDll4^{KO} mice were irradiated at day 0 and induced with tamoxifen starting at day 3 for 5 consecutive days. **(B)** eDll4^{OE} mice were induced with doxycycline in their drinking water 7 days before sub-lethal irradiation. Both experimental groups were sacrificed at days 8 or 26 following irradiation. Non-irradiated animals were induced at the same time-points and sacrificed at the equivalent of 26 days after irradiation.

4.3.3. Flow cytometry

To sort BM endothelial cells, total BM mononuclear cells from 2 mice were pooled. To achieve this, BM cells were flushed with PBS 2mM EDTA and enriched for the Lineage$^-$ population by magnetic cell sorting using a Lineage antibody cocktail coupled to magnetic beads (MACS system, Miltenyi Biotec). Lin$^-$CD105$^+$ were sorted using anti-CD105 (MJ7/18) PE (eBiosciences) antibody. All sortings were performed with an Aria Cell Sorter equipped with FACS Diva 6.2 Software (BD Biosciences). Dead cells and debris were excluded by FSC, SSC and 7AAD (7-Amino-Actinomycin D) cell viability solution (BD Biosciences) staining profiles, and sorted cells were collected into TRIzol Reagent (Invitrogen).

4.3.4. Immunostaining and imaging

Femurs were formalin-fixed, decalcified with Calci Clear (National Diagnostics) for 24h, and processed for routine histopathology. Immunostaining for endothelial cells and megakaryocyte markers was performed on 3µm slices. Sections were incubated with the primary antibodies listed on **Table 4.II** for 1 hour at room temperature. Femurs for

immunohistochemistry were stained according to the visualization system manufacturer's instructions and counterstained with hematoxylin. The slides were then analyzed using a Leica DM2500 microscope and all images were acquired with the 40x objective. Sections for immunofluorescence were incubated with secondary Alexa Fluor 488 and Alexa Fluor 594 antibodies for 1 hour at room temperature and DNA was stained with DAPI Vectashield mounting medium (H-1200, Vector Laboratories). Imaging was performed using a Zeiss LSM 510 META microscope and images were acquired with the 40x water immersion objective. The number of vessels or cells stained by each marker was quantified and is shown as a mean of 10 representative images of individual mouse femurs.

For B cell imaging, femurs were fixed overnight in 4% paraformaldehyde (PFA) at 4ºC with constant shaking, decalcified in 0,5% EDTA pH 7.5 for 3-4 days at 4ºC and imbedded in cutting temperature compound (OCT) (Tissue-Tek) at -20ºC. Frozen sections of 20 µm were obtained using a LEICA CM 3050 S Cryostat. Sections were permeabilized and blocked in PBS containing 1% BSA and 0,1% Triton X-100, and stained with B220 and VE-Cadherin antibodies overnight. The tissues were incubated with secondary antibodies for 2h and mounted in DAPI Vectashield mounting medium (H-1200, Vector Laboratories). Images were acquired using a Zeiss LSM 510 META microscope and reconstructed in three dimensions for B cell localization analysis with the LSM 510 software. The number of B cells in each category was quantified and is shown as a mean of 5 representative stacks (10 µm thickness each) of individual mouse femurs.

Table 4.II. Antibodies list.

Antigen	Antigen retrieval	Dilution	Brand
B220	HIER, Tris-EDTA pH 9	1:100	BD 550286
CD105	HIER, Tris-EDTA pH 9	1:150	R&D AF1320
Dll4	PIER, Pepsin	1:100	R&D AF1389
VE-Cadherin	PIER, Pepsin	1:150	R&D AF1002
VEGFR2	HIER, Tris-EDTA pH 9	1:100	R&D AF644
VEGFR3	HIER, Tris-EDTA pH 9	1:100	BD 552857
vWF	PIER, Pepsin	1:300	DAKO A0082
Secondaries for immunohistochemistry			
Anti-Goat, peroxidase	---	Ready-to-use	VectorLabs MP-7405
Anti-Rat, peroxidase	---	Ready-to-use	VectorLabs MP-7444

4.3.5. RNA isolation and quantitative PCR

RNA was extracted according to Invitrogen's instructions for TRIzol Reagent. Reverse transcription was performed with SuperScript II (Invitrogen), using Random Primers (Sigma) and according to the manufacturer's protocol. Quantitative PCR was performed with Power SYBR Green PCR Master Mix (Roche) on a ViiA™ 7 Real-Time PCR System (Life Technologies). The sequences of the oligonucleotides used are included in **Table 4.III**. A primer concentration of 180nM was found to be optimal in all cases. Amplification of beta-2-microglobulin (*B2m*) was used for sample normalization of the mouse samples; hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) together with *B2M* amplification were used for human samples normalization.

Table 4.III. Primers for qPCR.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
m*B2m*	TCACGCCACCCACCGGAGAA	TGTGAGGCGGGTGGAACTGTG
m*Cxcl12*	GCCAACGTCAAGCATCTGAAAA	TCTTCAGCCGTGCAACAATC
m*Cxcr4*	GGGACATCAGTCAGGGGGAT	CTATCGGGGTAAAGGCGGTC
m*Il7*	TTTCTGCCCAATGATCTTCC	CAGGGGACCTAGAGGAAAGG
m*Scf*	GGCAAATCTTCCAAATGACTATATGA	GCCAACAATGACTAGGCAAAACA
m*Thpo*	AAGCTCCGGGCGAGATGT	AAGCTCCGGGCGAGATGT
m*Vcam1*	GCCTCAACGGTACTTTGGATA	TGGAGTCACCGATTTGAGCAAT
h*B2M*	TCGCTCCGTGGCCTTAGCTGT	CTTTGGAGTACGCTGGATAGCCTCC
h*HPRT*	CTTTGCTTTCCTTGGTCAGGCAGT	CGTGGGGTCCTTTTCACCAGCAA
h*CXCL12*	ATGCCCATGCCGATTCTT	GCCGGGCTACAATCTGAAGG
h*CXCR4*	TGACTTGTGGGTGGTTGTGT	CCAGGCAGGATAAGGCCAAC

4.3.6. Cell culture and treatment

Human umbilical cord vein endothelial cells (HUVECs) (Clonetics, Lonza) were cultured in 24-well plates (Corning) at the density of $5x10^4$ cells/mL in EBM-2 supplemented with EGM-2 Single-Quots, 2mg/mL BBE (Lonza) and 1% heat-inactivated fetal bovine serum (FBS) (Gibco). To inhibit Notch1 and Delta-like 4 (Dll4)-mediated Notch signaling, cells were treated with 5µg/mL of neutralizing anti-human Notch1 antibody (MHN1-128) (Sekine et al. 2012) or 20µg/mL of anti-human Dll4 antibody (MHD4-46) (Sunamura & Yagita 2008) (kindly provided by Dr. Hideo Yagita), 24 hours after plating and for 24 hours. Equal volume of PBS was added to the control wells. After 24 hours of culture,

cells were washed with PBS and collected into TRIzol Reagent (Invitrogen) for mRNA analysis.

4.3.7. Chemotaxis assay

$CD34^+$ cells were isolated from buffy coats obtained from the Portuguese Blood Institute. Briefly, the content of one buffy coat was diluted in an equal volume of PBS and then layered over LymphoSep-Lymphocyte Separation Medium (MP Biochemichals). Tubes were centrifuged for 30 minutes at 1800 rpm at room temperature and the interface containing the peripheral blood mononuclear cells (PBMC) was collected and treated with Red Blood Cell Lysis Buffer (Biolegend) for 15 minutes in the dark. $CD34^+$ cells were then purified from the PBMCs using the human CD34 MicroBead Kit (Miltenyi Biotec) according to the manufacturer's protocol. $CD34^+$ cells were treated with 1µM of CXCR4 inhibitor AMD3465 (Tocris) in RPMI 10% FBS for 30 minutes at 37ºC. A total of $1x10^5$ treated cells in a volume of 100µL were added to the top chamber of 6.5mm diameter Transwell culture inserts (Corning) with a pore size of 8µm. Inserts were placed in wells containing 600µL of either RPMI 10% FBS, RPMI 10% FBS with 50ng/mL SDF1α (R&D Systems) or $1x10^5$ preplated human umbilical vein endothelial cells (HUVECs). 12 hours after plating, HUVECs were treated with 20µg/mL of anti-human Dll4 neutralizing antibody for 24 hours (MHD4-46, kindly provided by Dr. Hideo Yagita) (Sunamura & Yagita 2008). HUVECs were then washed and 600µL RPMI 10% FBS was added. Chemotaxis assays were performed at 37°C for 8 hours. Cells that migrated were counted in duplicate from each triplicate using a Burker hemocytometer (Blau Brand).

4.3.8. Statistical analysis

Data processing was carried out using Graph Pad Prism 6 Software and statistical analysis was performed using unpaired two-tailed Student's t test. Results are expressed as mean ± standard deviation. P values of <0.05 were considered statistically significant.

4.4. RESULTS

4.4.1. At steady state, endothelial-specific Delta-like 4 modulation does not affect the BM vascular niche.

The Notch signaling pathway has been shown to play a critical role in both vascular development and postnatal angiogenesis (Phng & Gerhardt 2009; Roca & Adams 2007). Notch ligand Dll4, which is particularly confined to endothelial tip cells, is essential for Notch function in such settings (Ribatti & Crivellato 2012). To examine the involvement of endothelial-specific Dll4 (eDll4) in the maintenance and function of the BM vascular niche, we took advantage of previously established conditional Dll4 loss- and gain-of-function mouse models (described in Section 4.3.2). Mice were induced at 8 weeks of age, for five consecutive days, and sacrificed 19 days after induction, a time-point at which we characterized the BM vascular niche using 4 different vascular markers: VEGFR2, VEGFR3, VE-Cadherin and CD105 (Endoglin). CD105, VE-cadherin and VEGFR2 have been extensively used to identify BM endothelial cells (Kunisaki et al. 2013; Nombela-Arrieta et al. 2013) and VEGFR3 has been described as a specific marker of BM sinusoidal endothelial cells (SECs) (Kunisaki et al. 2013; Hooper et al. 2009).

We found that the numbers of vessels stained for the different markers did not change in either mutant mice (**Figure 4.2**), suggesting that, despite the hematopoietic changes found in mice at steady state (described in Section 3.4.2), the identity and number of vessels in the BM was not modified by the modulation of eDll4 at steady-state.

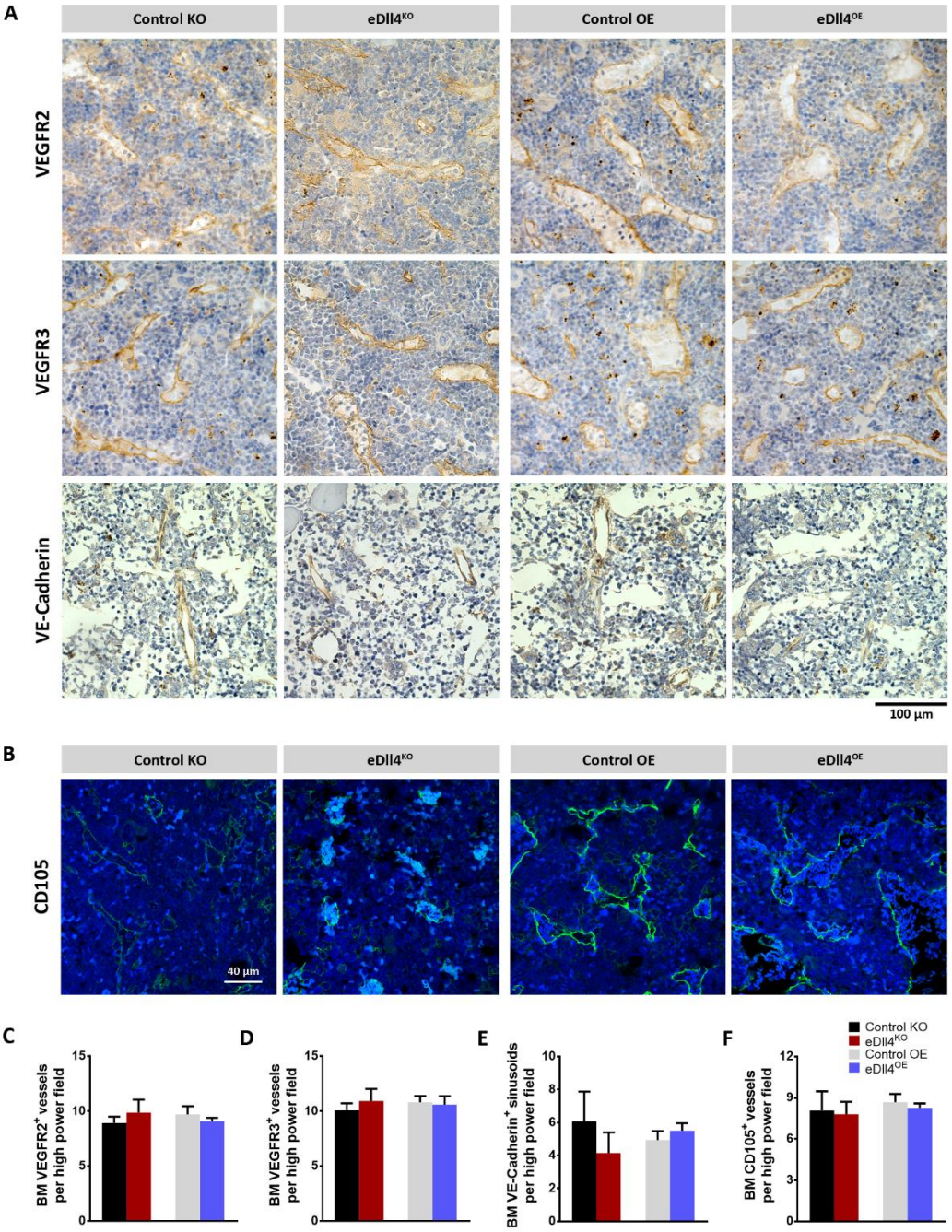

Figure 4.2. Modulating endothelial Dll4 does not affect the BM vascular niche at steady state

(A) Representative immunohistochemistry images of femoral bone marrows stained with anti-VEGFR2, anti-VEGFR3 and anti-VE-Cadherin and counterstained with hematoxylin. **(B)** Representative immunofluorescence images of femur bone marrows stained with anti-CD105 (green) and counterstained with DAPI (blue) **(C-F)** Quantification of the total number of vessels stained with each endothelial maker show that eDll4 modulation does not affect the total number of vessels or sinusoids at steady state, as accounted by **(C)** VEGFR2, **(D)** VEGFR3, **(E)** VE-Cadherin and **(F)** CD105 staining. Results are represented as a mean of 10 representative images from individual mouse femurs (n=3, data is represented as mean ± SD).

4.4.2. Sub-lethal irradiation causes a modulation in BM vessel identity in early stages of recovery

After myeloablation with cytotoxic agents or exposure to radiation, the BM sinusoids become severely damaged (H. G. Kopp et al. 2005; Li et al. 2008; Hooper et al. 2009; Li et al. 2010) and the sinusoidal endothelial cells that survive irradiation proliferate to replace the injured cells, maintaining the structural integrity of the BM vascular niche (Li et al. 2008). Therefore, we sub-lethally irradiated eDll4^{KO} and eDll4^{OE} mice with a low dose of irradiation (300rad) to induce a mild vascular regression in the BM (Hooper et al. 2009) and analyze the role of eDll4 in BM vessel rearrangement and endothelial cell proliferation. We characterized the BM vascular niche 8 and 26 days after sub-lethal irradiation, accounting for an early and late stage of BM recovery following myelosuppression (**Figure 4.3 and Figure 4.4**).

By day 8 after sub-lethal irradiation, the BM vasculature of the mouse mutants revealed a significant increase in VEGFR2 positive vessels in eDll4^{KO} mice and a decrease in eDll4^{OE} mice (**Figure 4.3A and C**). These alterations are consistent with previous reports showing that retinal vessels of early postnatal Dll4$^{+/-}$ mice have an upregulation of VEGFR2 (Suchting et al. 2007) and that Dll4-Notch signaling downregulates VEGFR2 in cultured endothelial cells (Williams et al. 2006). Furthermore, eDll4^{KO} mice exhibited increased VE-Cadherin positive vessels and an apparently augmented expression of this marker when compared to VE-Cadherin^{+} vessels in the respective control mice (Control KO) (**Figure 4.3B and E**). The total number of endoglin (CD105) and VEGFR3 expressing vessels did not change in either mouse mutant (**Figure 4.3A, B, D and F**).

Such changes in vessel number, however, were completely reversed by day 26 post myeloablation. VE-cadherin positive vessels in eDll4^{KO} decreased to similar levels as the ones found in non-irradiated mice (**Figure 4.4A and B**) whereas the number of CD105^{+} vessels remained similar throughout recovery and between the different mouse models (**Figure 4.4A and C**).

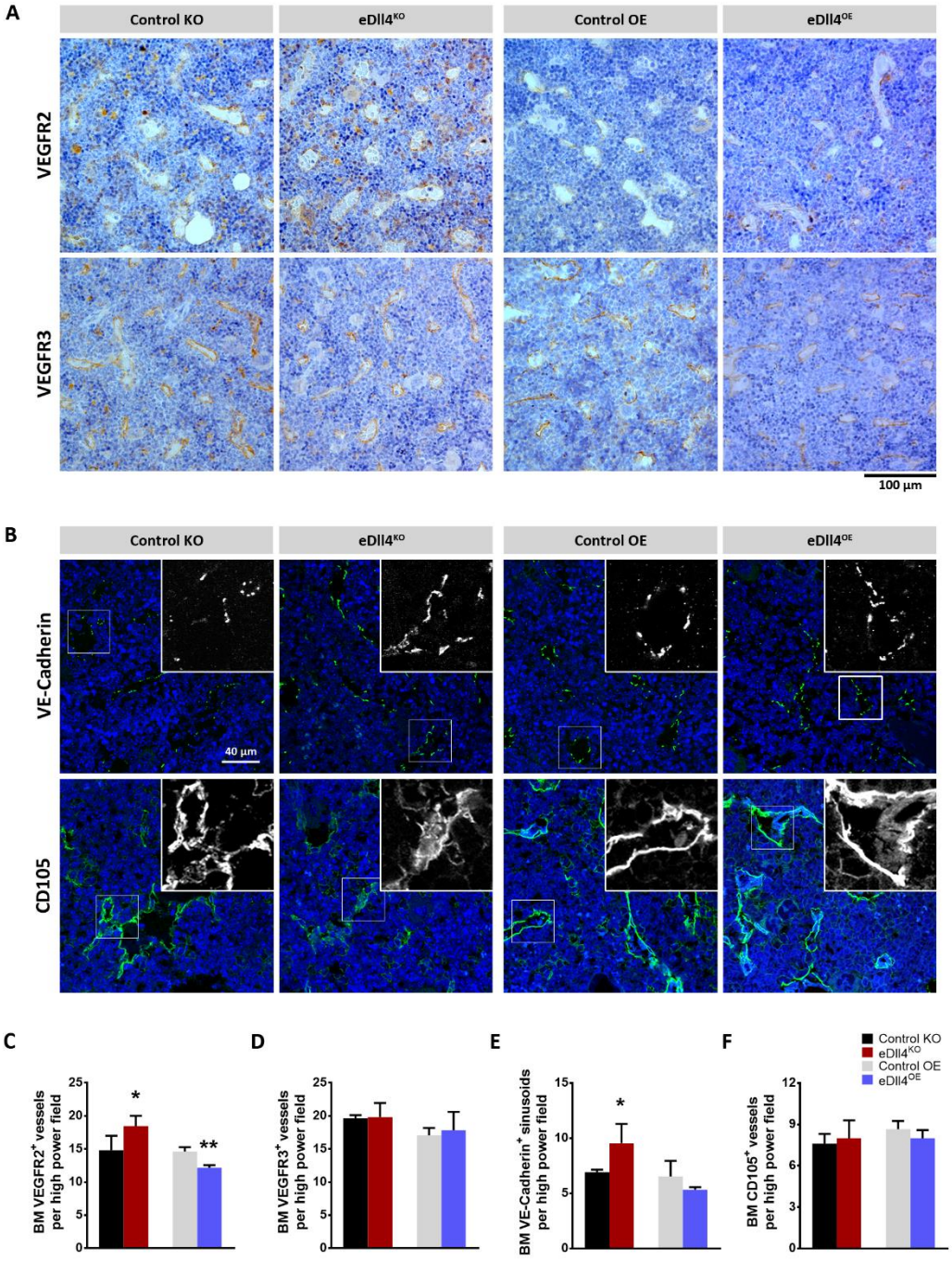

Figure 4.3. Endothelial Dll4 modulation induces changes in VEGFR2 and VE-Cadherin vessel number 8 days after irradiation

(A) Representative immunohistochemistry images of femoral bone marrows stained with anti-VEGFR2 and anti-VEGFR3 and counterstained with hematoxylin. **(B)** Representative immunofluorescence images of femur bone marrows stained with anti-CD105 and anti-VE-Cadherin (green) and counterstained with DAPI (blue). Insets are magnified 2x. **(C-F)** Quantification of the total number of vessels at 8 days post-irradiation stained with each endothelial maker show that eDll4 levels negatively correlate with the number of VEGFR2 and VE-Cadherin positive vessels, but eDll4 modulation does not affect the number of CD105 and VEGFR3 positive vessels. Results are represented as a mean of 10 representative images from individual mouse femurs (n=4, data is represented as mean ± SD; * p<0,05)

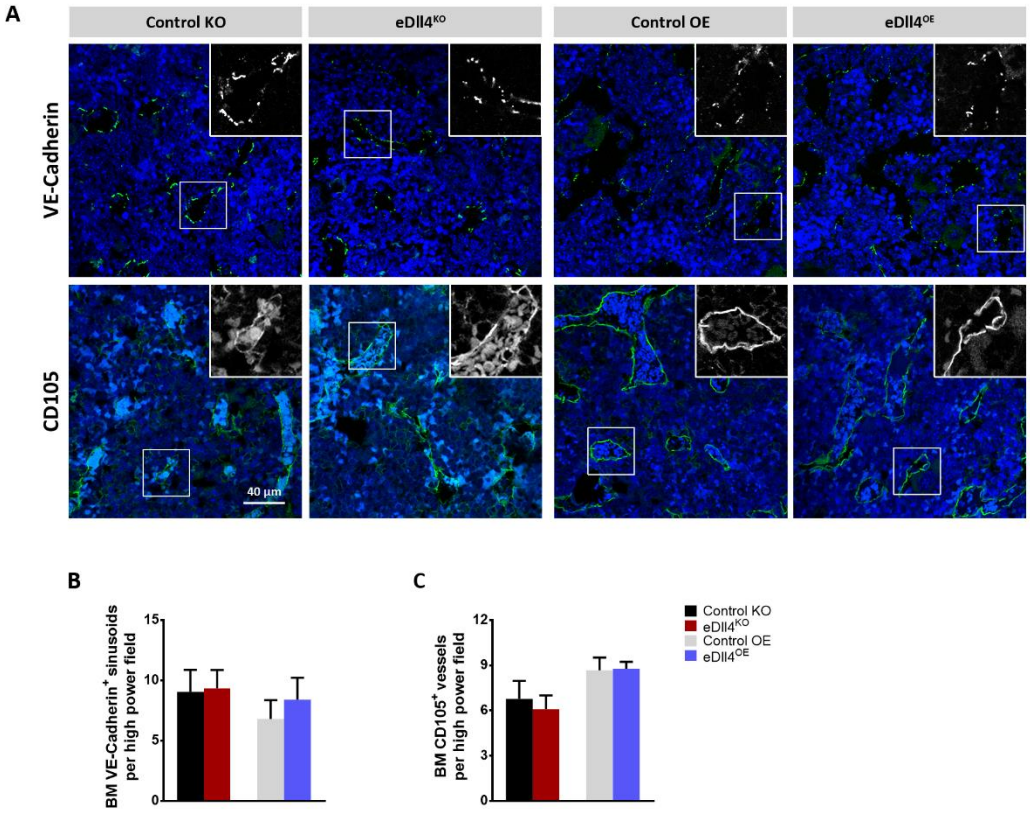

Figure 4.4. Normal vessel number is restored by 26 days after irradiation

(A) Representative immunofluorescence images of femur bone marrows stained with anti-CD105 and anti-VE-Cadherin (green) and counterstained with DAPI (blue). Insets are magnified 2x. **(B-C)** Quantification of the total number of vessels 26 days after irradiation stained with VE-Cadherin and CD105. Results are represented as a mean of 10 representative images from individual mouse femurs (n=4, data is represented as mean ± SD; * p<0,05).

To understand whether endothelial Dll4 knockout and overexpression was maintained throughout recovery, we looked at Dll4 positive vessels in both time-points and analyzed Dll4 distribution in the BM vascular niche (**Figure 4.5**). We found that Dll4 staining at the 8d time-point already revealed an efficient induction of eDll4 modifications (**Figure 4.5A**) but this modulation of eDll4 levels was even more evident by day 26 of recovery (**Figure 4.5B**), where we found a significant reduction in Dll4-positive vessels in eDll4^{KO} mice compared to Control KO mice (2,12 ± 1,32 and 4,90 ± 0,47, respectively) and significant higher number of these vessels in eDll4^{OE} mice (8,65 ± 0,81) than in Control OE mice (6,33 ± 0,90). Importantly, in eDll4^{KO} mice, there was a clear reduction of Dll4 coverage when compared to the control counterparts. While in Control KO mice the majority of positive

vessels presented Dll4 staining lining the totality of the vessel lumen, eDll4^{KO} Dll4^{+} vessels showed a discontinuous Dll4 staining around their lumen (depicted in **Figure 4.5A** insets).

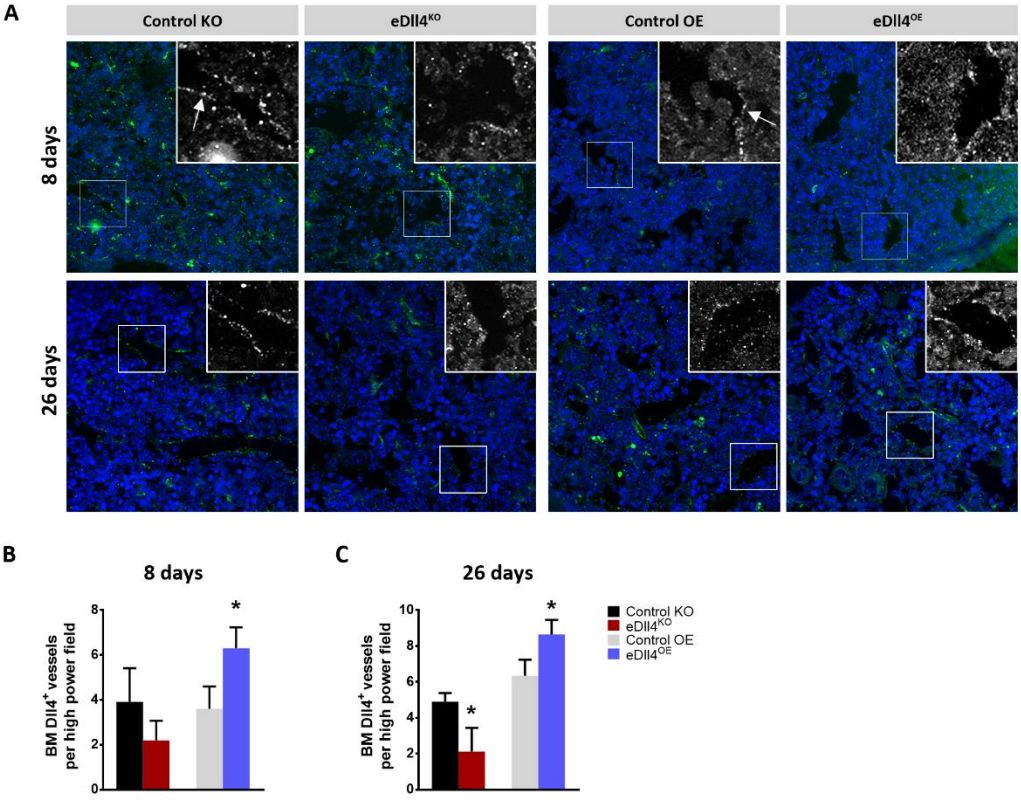

Figure 4.5. The number of Dll4-positive vessels correlates with eDll4 knockout and overexpression

(A) Representative images of Dll4 staining in each group at both 8 and 26 days after irradiation show that eDll4^{KO} mouse have less Dll4 coverage around the vessel lumen than the respective controls. Insets are magnified 2,5x. **(B)** Quantification of the total number of Dll4^{+} vessels 8 days after irradiation revealed a significant decrease in eDll4^{KO} mice. **(C)** Total number of Dll4-positive vessels was still decreased 26 days post-irradiation in eDll4^{KO} mice, and significantly increased in eDll4^{OE} mice. Data are means ± SD of 4 mice per experimental group (* $p<0.05$).

4.4.3. eDll4 affects B cell and megakaryocyte localization within the BM vascular niche following myelosuppression

The BM vascular niche is required for hematopoietic stem cell and hematopoietic progenitor cell maintenance and differentiation (Butler, Nolan, et al. 2010; Kobayashi et al. 2010; He et al. 2014; Kunisaki et al. 2013; Ding et al. 2012; Ding & Morrison 2013; Avecilla et al. 2004; Hamada et al. 1998). To understand how modulation of the BM vascular niche could account for the hematopoietic changes described in **Chapter 3**, we first investigated whether the localization of the hematopoietic cells was affected in the different mouse

mutant lines throughout recovery. To do so, we used a B220 antibody to stain B lymphocytes from early pro-B stages to mature B cells (Nagasawa 2006). BM vessels were labeled with VE-Cadherin, which is specific of the type of vessels were Dll4 is knocked out in eDll4^{KO} mice. B cells were then "divided" into 3 different categories, taking into account their localization relative to the VE-Cadherin^{+} vessels: inside, attached or away from VE-Cadherin positive vessels (**Figure 4.6A and B**).

By day 8 after irradiation, we did not observe differences in the percentage of B cells inside the vessels, both in eDll4^{KO} and in eDll4^{OE} mutant mice. However, we found that eDll4^{KO} mice had a significantly lower percentage of cells attached to the base of VE-Cadherin^{+} vessels (10,85% ± 1,05) when compared to Control KO mice (17,44% ± 0,69), and that the opposite was true in eDll4^{OE} mice (20,21% ± 1,94 in Control OE and 24,14% ± 1,54 in eDll4^{OE} mice). Consistently, eDll4^{KO} exhibited a significantly increased percentage of B220

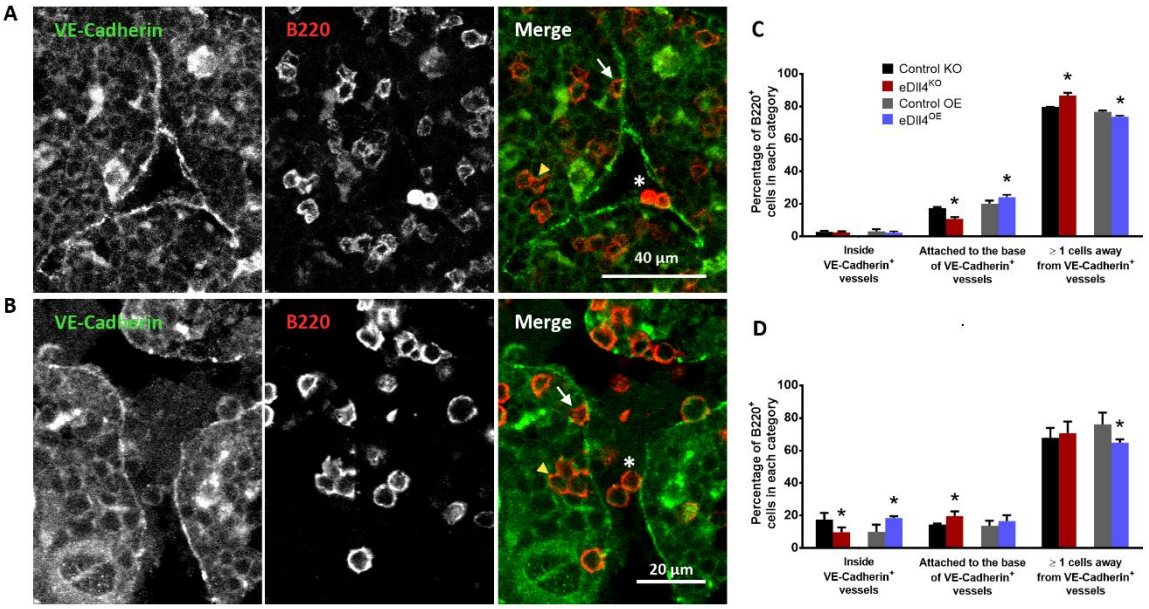

Figure 4.6. B lymphocyte localization relative to the BM vascular niche is affected by eDll4 levels

(A-B) Representative images showing B220^{+} B cell (red) localization within the BM vascular niche (green). B cells were divided into three sub-groups, considering their position relative to VE-Cadherin^{+} vessels: B cells inside VE-Cadherin^{+} vessels (asterisks), B cells in close contact with VE-Cadherin^{+} vessels (white arrows) and B lymphocytes that are more than one cell away from any VE-Cadherin^{+} vessel (yellow arrowheads). **(C-D)** Quantification of the percentage of B cells in each category shows that **(C)** by day 8 into recovery, knocking out Dll4 decreases the number of B cells contacting VE-Cadherin^{+} vessels whereas overexpressing endothelial Dll4 increases the percentage of B cells included in that category. **(D)** 26 days after irradiation, the number of B cells inside VE-Cadherin vessels was decreased in eDll4^{KO} and increased in eDll4^{OE} mice. Data are means ± SD of 3 mice per experimental group (* $p<0.05$).

cells away from any VE-Cadherin^{+} vessel, and eDll4^{OE} had a lower percentage of cells included in this category (**Figure 4.6C**).

In the late stage of BM recovery following myeloablation (26d), however, B cells close to VE-Cadherin vessels were increased in eDll4^{KO} mice and eDll4^{OE} mice showed similar numbers to Control OE. Furthermore, the trend observed in both mouse mutants in B cells attached to VE-Cadherin^{+} vessels by day 8, was similar to the one observed in B cells inside the vessels 26 days post-irradiation (**Figure 4.6D**), suggesting that B cell mobilization into the bloodstream correlates with their localization close to BM sinusoids in earlier time points. Accordingly, eDll4^{KO} mice had lower levels of B lymphocytes inside BM sinusoids than Control KO mice (9,76% ± 2,95 and 17,67% ± 3,94, respectively) whereas eDll4^{OE} mice showed higher levels of B cells in this category (18,52% ± 1,14), compared to Control OE mice (10,11% ± 4,34).

Remédio and colleagues have previously shown that blocking Dll4 systemically significantly increased the total number of megakaryocytes (MKs) in the BM (Remédio et al. 2012), consistent with the findings that Dll4/Notch signaling inhibited MK differentiation (Poirault-Chassac et al. 2010). To understand whether eDll4 could affect MKs in the BM, we used an antibody against von Willebrand Factor (vWF), a commonly used marker for megakaryocytes (Chuang et al. 2000) and analyzed MK number and localization 8 and 26 days after sub-lethal irradiation (**Figures 4.7 and 4.8**).

Surprisingly our data showed that, by day 8 into recovery, eDll4^{OE} and Control OE mice had no differences in MK number but eDll4^{KO} mice had significantly less MKs than Control KO mice (**Figure 4.7A and B**). This decrease in MK number was accompanied by a reduction in the percentage of MKs in close contact with VE-Cadherin^{+} vessels (**Figure 4.7A and C**), suggesting that MK counts in the BM correlate with their localization in the BM vascular niche. Twenty-six days after myelosuppression, however, the number of MKs in the bone marrow of both eDll4^{KO} and eDll4^{OE} mice were increased when compared to the respective controls (**Figure 4.8A and B**), without significant differences in their localization (**Figure 4.8A and C**).

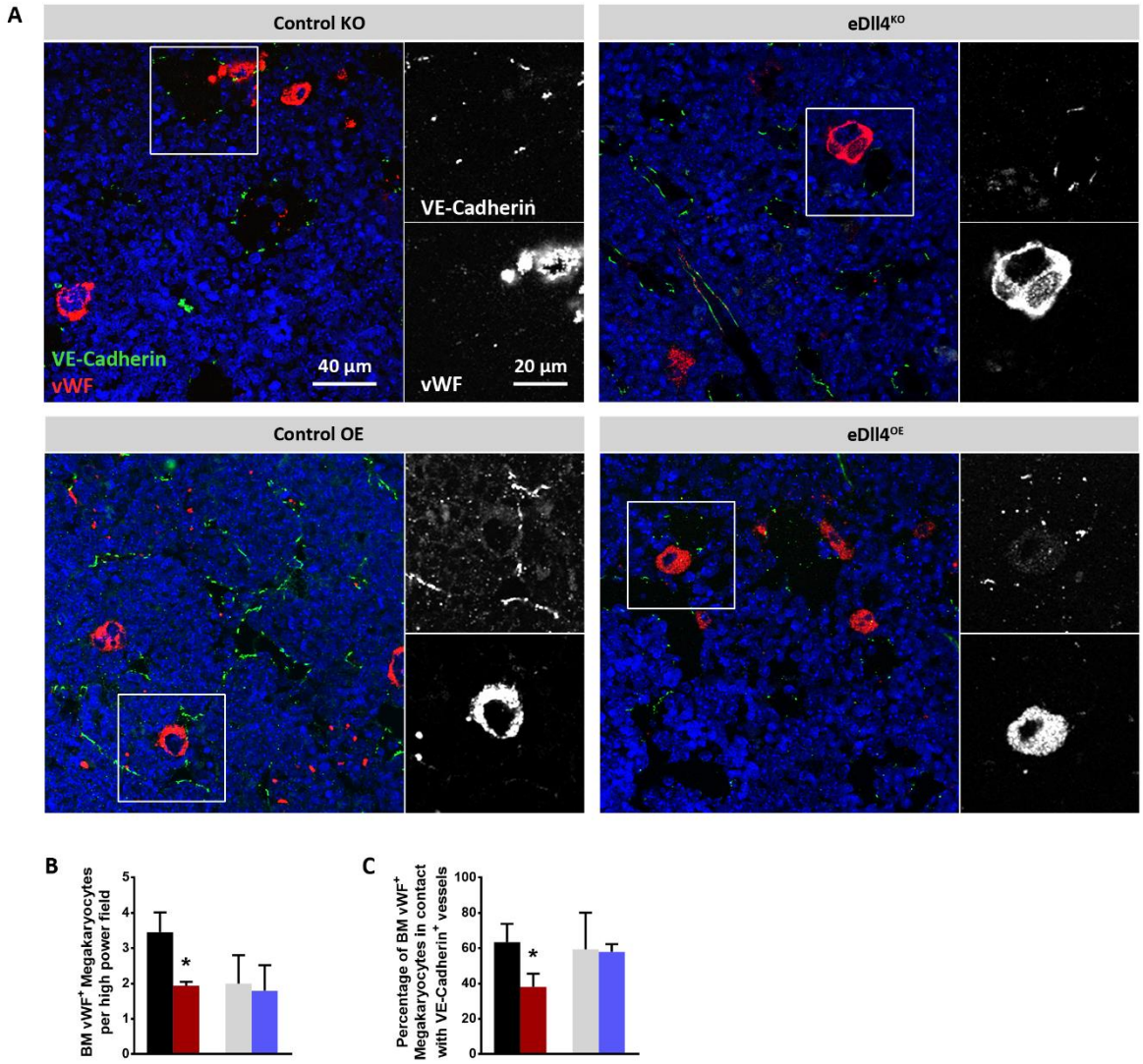

Figure 4.7. eDll4 knockout decreases megakaryocyte number and localization near BM sinusoids upon myeloablation

(A) Representative images of megakaryocyte (red) localization relative to VE-Cadherin^{+} vessels (green). Insets are magnified 1.75x. **(B)** MK quantification shows that eDll4^{KO} mice have decreased numbers of MKs in the BM but eDll4^{OE} and Control OE mice have similar MK numbers. **(C)** Quantification of the percentage of MKs in close contact with VE-Cadherin^{+} vessels also shows a decrease in eDll4^{KO}, but not in eDll4^{OE} mice. Data are means ± SD of 4 mice per experimental group (* $p<0.05$).

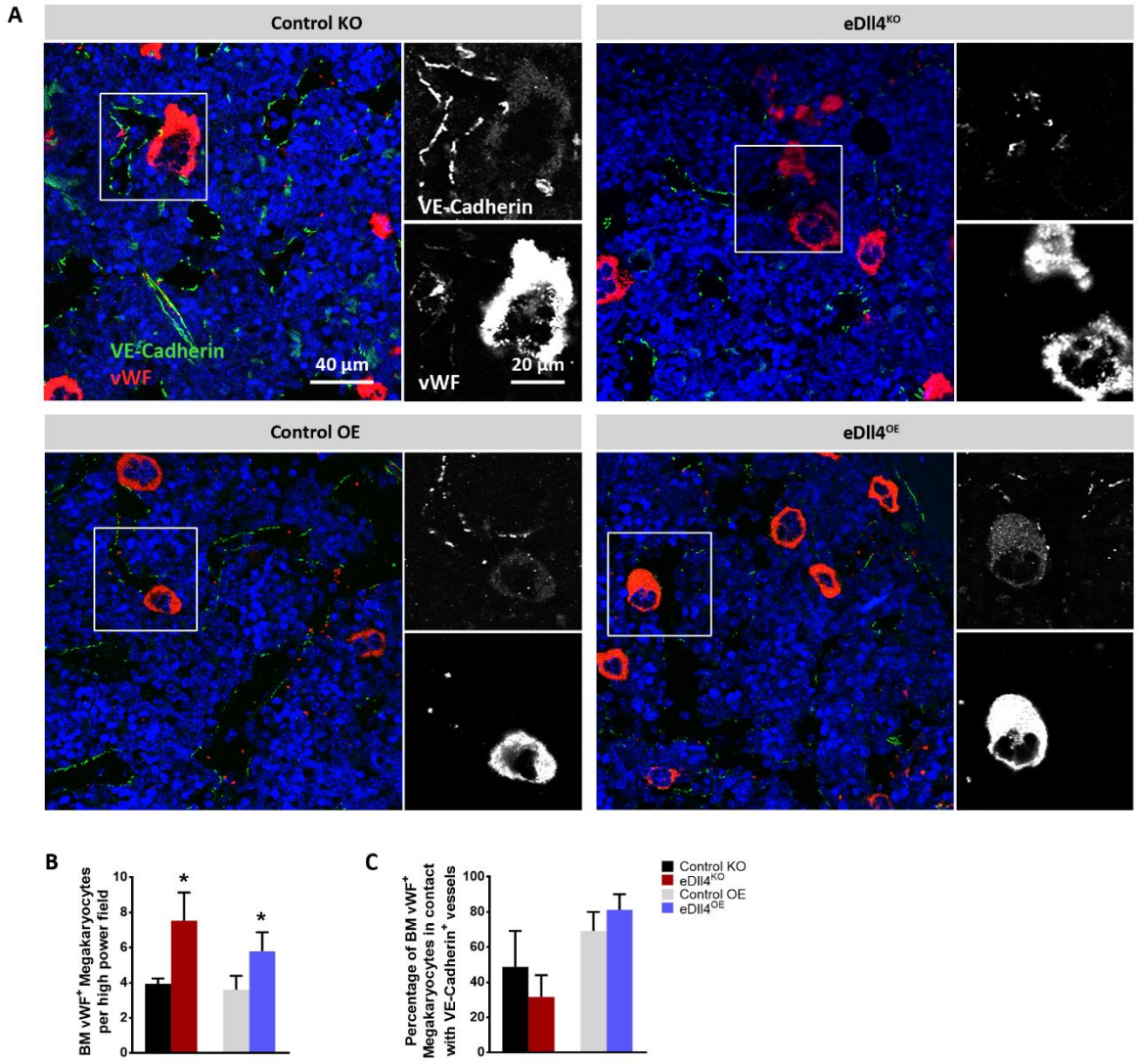

Figure 4.8. Megakaryocyte number is increased by both eDll4 knockout and overexpression in later stages of recovery, but MK localization relative to BM sinusoids is not affected

(A) Representative images of megakaryocyte (red) localization relative to VE-Cadherin^{+} vessels (green). Insets are magnified 1.75x. **(B)** Quantification of MK number shows an increase in both eDll4^{KO} and eDll4^{OE} mice, compared to the respective controls. **(C)** The percentage of MKs near BM sinusoids was quantified and revealed no differences in both mouse mutants. Data are means ± SD of 4 mice per experimental group (* $p<0.05$).

4.4.4. eDll4 modulates the expression of "angiocrine genes" throughout recovery

Having demonstrated that eDll4 affected BM recovery (**Chapter 3**) and the localization of some subsets of hematopoietic cells, we hypothesized that the hematopoietic changes observed in our mice models derived from a modulation in the expression of the so-called "angiocrine genes". Those genes are expressed by endothelial cells and determine the instructive role of the vascular niche, essential in hematopoietic stem and lineage-specific compartments reconstitution after an acute injury to the BM, such as exposure to radiation (Kobayashi et al. 2010). We isolated BM endothelial cells (Lin^-CD105^+) from both mouse models and performed a qPCR analysis on angiocrine genes known to affect B cell and MK differentiation and mobilization. As shown in **Figure 4.9A**, 8 days after sub-lethal irradiation, endothelial cells from $eDll4^{KO}$ mice had a 72% decrease in *Cxcl12* expression, and a 4.4 fold increase in *Scf* expression, whereas both genes were downregulated in

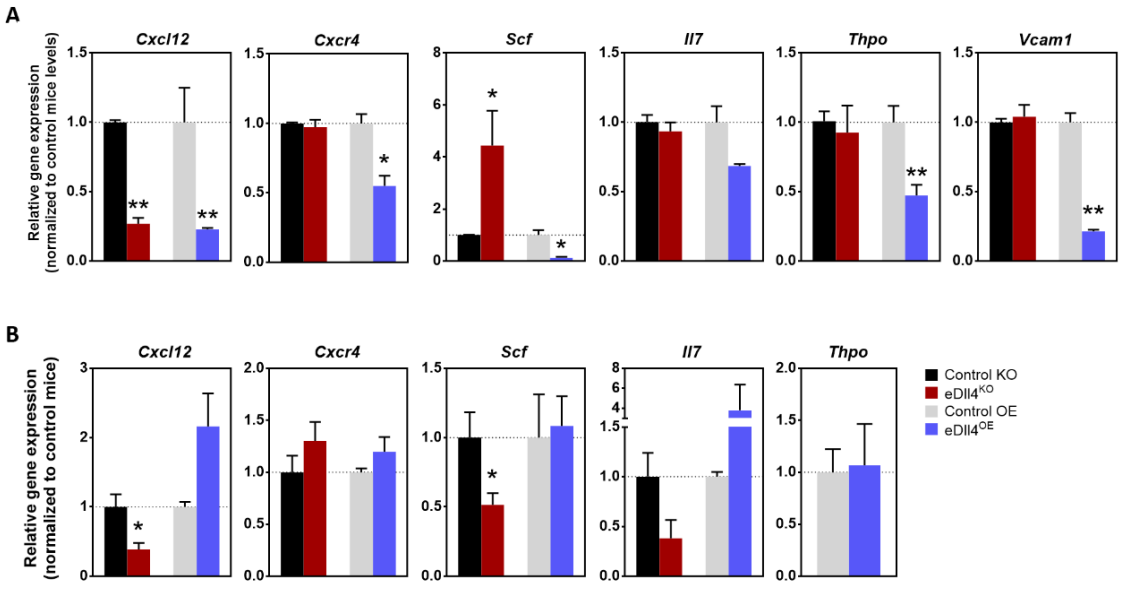

Figure 4.9. Endothelial-specific Dll4 modulates angiocrine gene expression in the BM microenvironment throughout recovery

(A) Angiocrine gene modulation in $CD105^+$ cells 8 days after sub-lethal irradiation was assessed by quantitative PCR. mRNA analysis showed that Cxcl12 is downregulated and *Scf* is upregulated in BM endothelial cells from $eDll4^{KO}$ mice. A significant reduction in *Cxcl12*, *Cxcr4*, *Scf*, *Thpo* and *Vcam1* expression was observed in $CD105^+$ cells isolated from $eDll4^{OE}$ mice. **(B)** Angiocrine gene expression in $CD105^+$ cells 26 days after myelosuppression was also assessed. BM endothelial cells isolated from $eDll4^{KO}$ mice exhibited a decrease in *Cxcl12*, *Scf* and *Il7* expression levels, whereas $eDll4^{OE}$ mice showed upregulation of *Cxcl12* and *Il7*. Data are means ± SD of 3 pooled samples per experimental group (* p<0,05, ** p<0,01).

eDll4^{OE} mice (77% and 87% decrease, respectively). *Cxcr4* and *Il7*, which similarly to Scf and Cxcl12 are involved in B cell differentiation and mobilization (Nagasawa 2006), were also decreased in eDll4^{OE} mice. Endothelial cells from eDll4^{OE} mice also exhibited reduced *Thpo* (53% decrease), which is a primary regulator of MK and platelet production (Murone et al. 1998), and *Vcam1* expression (79% reduction compared to Control OE mice), which is required for leukocyte migration into and out of the BM (Koni et al. 2001; Leuker et al. 2001).

By day 26 post irradiation, angiocrine gene expression markedly diverged from what was observed at 8 days post-irradiation. Indeed, whereas *Cxcl12* expression remained lower in eDll4^{KO}, it was increased in eDll4^{OE} mice. *Scf* expression levels in eDll4^{OE} mice were similar to Control OE mice, but were significantly decreased in eDll4^{KO} (49% lower than Control KO mice). Furthermore, *Il7* was downregulated in eDll4^{KO} mice and upregulated in eDll4^{OE} mice. *Cxcr4* and *Thpo* were now similar in both mice mutants and respective controls (**Figure 4.9B**).

4.4.5. *In vitro* neutralization of Dll4 in HUVECs inhibits HSPC migration

Stromal-derived factor (SDF1α) has been shown to attract early stage B cell progenitors via the chemokine receptor CXCR4 (D'Apuzzo et al. 1997). Consistently, mice deficient in SDF1 or CXCR4 lack B lymphopoiesis and have severely impaired BM myelopoiesis (Nagasawa et al. 1996; Ma et al. 1999; Zou et al. 1998; Ma et al. 1998). Consistent with the decreased expression of *Cxcl12* (the gene coding for SDF1) in eDll4^{KO} endothelial cells, we found that *in vitro* treatment of HUVECs, a primary endothelial cell line, with either an anti-Dll4 or an anti-Notch1 neutralizing antibody also decreased *CXCL12* expression levels (**Figure 4.10A**). This treatment also increased *CXCR4* expression, consistent with reports showing that CXCR4 is downregulated by Dll4 (Williams et al. 2008).

To determine whether this alteration could account for the changes observed in B cell localization within the BM vascular niche, we inhibited Dll4-mediated Notch signaling in HUVECs and assessed B cell migration. To achieve that, we isolated human hematopoietic stem and progenitor cells (HPSC) using a CD34 marker and performed a chemotaxis assay with the anti-Dll4 treated HUVECs.

Cell migration experiments demonstrated that both SDF1α and HUVECs alone, known to produce SDF1α (Salvucci et al. 2002; Jin et al. 2013), induced migration of HSPCs (**Figure 4.10B**). Such effects were completely abolished when HSPCs were previously treated with a CXCR4 antagonist (AMD3465) that inhibits SDF1α-ligand binding. Remarkably, HUVECs treatment with anti-Dll4 neutralizing antibody induced a similar phenotype to that observed in AMD3465-treated HPSCs, and a combination of both treatments showed a cumulative effect, further inhibiting HSPC migration, suggesting that eDll4^{KO} BM endothelial cells hamper B lymphocyte migration towards the BM sinusoids through *Cxcl12* downregulation.

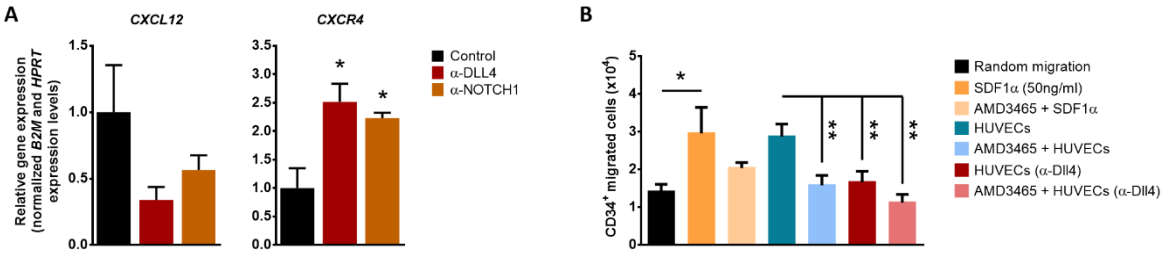

Figure 4.10. Dll4 neutralization in HUVECs downregulates CXCL12 and decreases HSPC migration

(A) Angiocrine gene modulation in HUVECs treated with an anti-Dll4 or anti-Notch1 neutralizing antibodies, showing that inhibition of the Notch signaling downregulates CXCL12 and upregulates CXCR4. **(B)** CD34^{+} cells (10^5) with or without AMD3465 (1μM) treatment were plated onto the upper chamber of transwell plates and exposed to 50ng/mL SDF1α or to 10^5 HUVECs pre-plated in the lower chamber with or without 20μg/mL α-Dll4 pre-incubation for 24 hours. The results are expressed as means ± SD of the total number of migrating cells (* $p<0.05$; ** $p<0.01$).

4.5. DISCUSSION

In this Chapter, we report the endothelial modifications that occur upon endothelial-specific modulation of the Notch ligand Dll4 expression levels in the BM microenvironment. Dll4 function in endothelial cells has been widely studied *in vivo*, particularly in retinas and tumor models, (Noguera-Troise et al. 2006; Lobov et al. 2011; Hellström et al. 2007; Lobov et al. 2007; Suchting et al. 2007; Jakobsson et al. 2010; Roca & Adams 2007) and *in vitro*, mainly in primary endothelial cells (HUVECs) (Williams et al. 2006; Estrach et al. 2011), but its specific function in BM endothelial cells had not been objectively addressed until

recently, where the effects of a systemic blockade of Dll4 in the bone marrow vascular niche and hematopoiesis were described (Remédio et al. 2012).

Our data shows that both knocking out Dll4 in VE-Cadherin^{+} cells and overexpressing Dll4 in Tie2^{+} cells in a setting of BM recovery after myeloablation affect the BM vascular niche in the early stages of recovery, but not in later stages or at steady state. Particularly, by day 8 following myeloablation, the number of VEGFR2^{+} vessels is inversely correlated with eDll4 levels and the VE-Cadherin-expressing vessels increase only in eDll4^{KO} mice, without quantitative changes in CD105^{+} and VEGFR3^{+} vessels.

VEGFR2 has been particularly linked to Dll4 in the endothelial tip/stalk cell selection process (Geudens & Gerhardt 2011; Hellström et al. 2007; Jakobsson et al. 2010). The cross talk between these two signaling pathways is evident, as the VEGF signaling through VEGFR2 in the retina tip cells induces Dll4 (Liu et al. 2003; Lobov et al. 2007; Suchting et al. 2007) and Dll4-Notch signaling downregulates VEGFR2 (Williams et al. 2006), which eventually leads to the stalk cell phenotype in the adjacent cells. VEGFR2 is the functional receptor that mediates VEGF-induced angiogenesis, vascular permeability and vascular remodeling (Ferrara et al. 2003) and has been shown to be essential for the regeneration of BM sinusoidal endothelial cells and consequent hematopoietic regeneration after sub-lethal irradiation (Hooper et al. 2009). VEGFR2 activation by VEGF is usually correlated with increased permeability as it causes rapid endocytosis of VE-Cadherin, disrupting the endothelial barrier function (Gavard & Gutkind 2006). Interestingly, despite the increase in VEGFR2^{+} vessel number in eDll4^{KO} mice, they exhibited both increased number and coverage of VE-Cadherin positive vessels. VE-Cadherin has also been linked to Dll4/Notch signaling as the Notch target gene *Slug* directly represses VE-Cadherin (Niessen et al. 2008). Consistently, Dll4-blocking antibodies lead to an increase in VE-Cadherin in mouse retinas (Bentley et al. 2014), which may explain why eDll4^{KO} mice have augmented number and coverage of BM VE-Cadherin^{+} sinusoidal vessels, despite the increase in VEGFR2-positive vessels. Our data thus suggests that eDll4^{KO} vessels are less permeable and consistently more stable, which might facilitate BM recovery. In eDll4^{OE} mice, however, the loss of VEGFR2 in BM sinusoids may affect the regeneration of endothelial cells following myeloablation and thus hamper hematopoietic recovery. This is supported by a variety of

studies showing that loss of either VEGFR2 or VE-Cadherin impairs normal BM recovery (Zeng et al. 2012; Hooper et al. 2009; Salter et al. 2009; Butler, Nolan, et al. 2010).

The reports of the role of the BM vascular niche in HSPC maintenance (Kunisaki et al. 2013; He et al. 2014; Gori et al. 2015; H.-G. Kopp et al. 2005; Kobayashi et al. 2010; Kubota et al. 2008; Hooper et al. 2009; Nombela-Arrieta et al. 2013) led us to hypothesize that these qualitative modulations in the BM vascular niche could be affecting hematopoiesis through differential localization of hematopoietic cells within the BM vascular niche. Concomitantly, we demonstrate that in the early stages of BM recovery (8 days), modulating eDll4 affects the percentage of B cells in close contact with VE-Cadherin^{+} vessels both in eDll4^{KO} and eDll4^{OE} mice, with VE-Cadherin vessels expressing putative higher amounts of Dll4 showing an increased percentage of B cells attached to them.

Particularly in eDll4^{KO} mice, the lower percentage of B cells in close contact with VE-Cadherin^{+} vessels might be due to a decrease in *Cxcl12* expression in the BM endothelial cells. Inhibition of Notch signaling in multiple myeloma cells has been shown to downregulate *Cxcl12* at the transcriptional and protein levels (Mirandola et al. 2013) and a similar mechanism may be responsible for downregulating this gene in HUVECs and BM endothelial cells. SDF1, and its major receptor CXCR4, regulate many aspects of hematopoietic cells, including their migration, survival and development (Dar et al. 2006). Studies *in vitro* have shown that SDF1 is chemotactic for cells that express CXCR4, including CD34^{+} HSPCs, lymphocytes, monocytes and megakaryocytes, and can promote their transendothelial migration (Ma et al. 1999; Marubini et al. 1999; Kawabata et al. 1999; D'Apuzzo et al. 1997; Hamada et al. 1998). Consistently, CXCR4/SDF1 signaling *in vivo* is essential for tissue retention of multiple cell types (Grunewald et al. 2006), as well as stem cell engraftment and BM reconstitution (Marubini et al. 1999; Juarez & Bendall 2004). Interestingly, as was previously demonstrated by Kimmel-Williams and colleagues, expression of Dll4 in endothelial cells decreases their migration to SDF1 by downregulating CXCR4 at the transcriptional and protein levels (Williams et al. 2008). Consistently, we have shown that *Cxcr4* expression negatively correlates with eDll4 levels, decreasing in eDll4^{OE} mice and increasing in HUVECs treated with a Dll4 neutralizing antibody.

Here, we demonstrate that treating CD34^{+} HSPCs with a CXCR4 inhibitor (AMD3465) leads to a decrease in CD34^{+} migration towards the SDF1 cue provided by HUVECs (Salvucci

et al. 2004; De La Luz Sierra et al. 2004). A similar reduction in $CD34^+$ cells migration was observed when HUVECs were treated with a Dll4 neutralizing antibody, showing that Dll4-mediated Notch signaling in endothelial cells is required for hematopoietic cell mobilization through SDF1 production. This suggests that although SDF1 is expressed in much higher levels by other BM cells, such as CXCL12-abundant reticular (CAR) cells and other perivascular mesenchymal stromal cells (Ding & Morrison 2013; Greenbaum et al. 2013), a decrease in endothelial-derived SDF1 is sufficient to reduce the attractive cues for hematopoietic cells and prevent B lymphocyte localization close to Dll4 knockout (VE-$Cadherin^+$) vessels.

The migration of hematopoietic cells towards the bloodstream requires them to cross the barrier composed by the BM sinusoidal endothelial cells. The reduction in endothelial *Cxcl12* expression levels by day 8 post-irradiation may therefore explain the decreased percentage of B cells inside VE-$Cadherin^+$ vessels and consequently in the peripheral blood observed by day 26 into recovery, despite their increased percentage in the BM. Interestingly, at this time-point *Cxcl12* levels are still reduced in $eDll4^{KO}$ mice, but the percentage of B cells close to VE-$Cadherin^+$ vessels is increased. This suggests that other factors, either endothelial or non-endothelial specific, may be involved in the recruitment of B cells towards the sinusoids. A similar effect may be occurring in $eDll4^{OE}$ mice, since the observed downregulation of endothelial *Cxcl12* in these mice by day 8 after myelosuppression was accompanied by an increase in the amount of B cells close to the sinusoids and a consequent increase in PB B lymphocytes, despite their lower levels in the BM. Interestingly, endothelial *Vcam1* downregulation does not seem to affect the migration of these cells into the periphery.

Furthermore, SDF1 downregulation could also account for the reduced percentage of BM B lymphocytes observed in $eDll4^{KO}$ mice 8 days post-irradiation, even though Dll4, the inhibitory signal for B cell maturation, was reduced and SCF, which acts synergistically with IL7 to promote pro-B cell proliferation (McNiece et al. 1991), was upregulated. SDF1 has also been shown to be crucial for proliferation and maintenance of B-lineage progenitors (Nagasawa 2006; Egawa et al. 2001; Nagasawa et al. 1996) and common lymphoid progenitors (Nie et al. 2008), and a disruption in the CXCR4/SDF1 axis leads to defects in B lymphopoiesis and BM myelopoiesis (Nagasawa et al. 1996; Ma et al. 1998; Zou et al. 1998).

We report additional alterations in MKs number and localization within the BM vascular niche upon eDll4 modulation. Thpo is the most potent cytokine for stimulation and maturation of MK progenitor cells. Knockout mice for Thpo or Thpo receptor (c-Mpl) show decreased MK size and have dramatically reduced levels of colony-forming units-megakaryocyte (CFU-MK) (de Sauvage et al. 1996; Gurney & de Sauvage 1996). Furthermore, although MK maturation and platelet production can be induced by the translocation of MK progenitor cells to the vicinity of BM sinusoids even in the absence of Thpo signaling, MK migration and adhesion to endothelial cells is dependent on chemokines like SDF1 and FGF4 (Avecilla et al. 2004; Niswander et al. 2014). It is therefore likely that the reduced platelet numbers found in eDll4^{OE} mice are caused by the reduction in both *Thpo* and *Cxcl12* expression levels, even though we did not find a decrease in MKs close to BM sinusoids. The downregulation of *Cxcl12* in eDll4^{KO} may have led to the decreased percentage of MKs in close contact with VE-Cadherin^{+} vessels. However, platelet release is dependent on the localization of MKs to sinusoidal BM endothelial cells, suggesting that other factors may be increasing platelet release to counteract the lower numbers of MKs in these animals.

Together, we demonstrate that endothelial Dll4 modulates the BM vascular niche identity 8 days following myeloablation, but not in later stages of recovery. This is accompanied by a modulation in the localization of B cells and MKs in the vicinity of BM sinusoids, in which lower levels of Dll4 correlate with decreased B/Mk cells in close contact with the sinusoid endothelial cells possibly as a consequence of endothelial-SDF1 downregulation.

4.6. REFERENCES

Avecilla, S.T. et al., 2004. Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis. *Nature medicine*, 10(1), pp.64–71.

Benedito, R. & Duarte, A., 2005. Expression of Dll4 during mouse embryogenesis suggests multiple developmental roles. *Gene Expression Patterns*, 5(6), pp.750–755.

Bentley, K. et al., 2014. The role of differential VE-cadherin dynamics in cell rearrangement during angiogenesis. *Nature cell biology*, 16(4), pp.309–21.

Butler, J.M. et al., 2010. Endothelial cells are essential for the self-renewal and repopulation of Notch-

dependent hematopoietic stem cells. *Cell Stem Cell*, 6(3), pp.251–264.

Chuang, S.S. et al., 2000. von Willebrand factor is the most reliable immunohistochemical marker for megakaryocytes of myelodysplastic syndrome and chronic myeloproliferative disorders. *American journal of clinical pathology*, 113(4), pp.506–11.

Cines, D.B. et al., 1998. Endothelial Cells in Physiology and in the Pathophysiology of Vascular Disorders. *Blood*, 91(10), pp.3527–3561.

Claxton, S. & Fruttiger, M., 2004. Periodic Delta-like 4 expression in developing retinal arteries. *Gene expression patterns : GEP*, 5(1), pp.123–7.

D'Apuzzo, M. et al., 1997. The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4. *European Journal of Immunology*, 27(7), pp.1788–1793.

Dar, A., Kollet, O. & Lapidot, T., 2006. Mutual, reciprocal SDF-1/CXCR4 interactions between hematopoietic and bone marrow stromal cells regulate human stem cell migration and development in NOD/SCID chimeric mice. *Experimental Hematology*, 34(8), pp.967–975.

Ding, B.-S. et al., 2014. Divergent angiocrine signals from vascular niche balance liver regeneration and fibrosis. *Nature*, 505(7481), pp.97–102.

Ding, B.-S. et al., 2011. Endothelial-Derived Angiocrine Signals Induce and Sustain Regenerative Lung Alveolarization. *Cell*, 147(3), pp.539–553.

Ding, B.-S. et al., 2010. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature*, 468(7321), pp.310–315.

Ding, L. et al., 2012. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*, 481(7382), pp.457–62.

Ding, L. & Morrison, S.J., 2013. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature*, 495(7440), pp.231–5.

Doan, P.L. et al., 2013. Tie2(+) bone marrow endothelial cells regulate hematopoietic stem cell regeneration following radiation injury. *Stem cells (Dayton, Ohio)*, 31(2), pp.327–37.

Duarte, A. et al., 2004. Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes and Development*, 18(20), pp.2474–2478.

Egawa, T. et al., 2001. The earliest stages of B cell development require a chemokine stromal cell-derived factor/pre-B cell growth-stimulating factor. *Immunity*, 15(2), pp.323–334.

Estrach, S. et al., 2011. Notch Signaling in Endothelial Cells.

Ferrara, N., Gerber, H.-P. & LeCouter, J., 2003. The biology of VEGF and its receptors. *Nature medicine*, 9(6), pp.669–676.

Gale, N.W. et al., 2004. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proceedings of the National Academy of Sciences of the United States of America*, 101(45), pp.15949–15954.

Gavard, J. & Gutkind, J.S., 2006. VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. *Nature cell biology*, 8(11), pp.1223–1234.

Geudens, I. & Gerhardt, H., 2011. Coordinating cell behaviour during blood vessel formation. , 4583, pp.4569–4583.

Gori, J.L. et al., 2015. Vascular niche promotes hematopoietic multipotent progenitor formation from pluripotent stem cells. *Journal of Clinical Investigation*, 125(3), pp.1–12.

Greenbaum, A. et al., 2013. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature*, 495(7440), pp.227–30.

Grunewald, M. et al., 2006. VEGF-induced adult neovascularization: Recruitment, retention, and role of accessory cells. *Cell*, 124(1), pp.175–189.

Gurney, a L. & de Sauvage, F.J., 1996. Dissection of c-Mpl and thrombopoietin function: studies of knockout mice and receptor signal transduction. *Stem cells*, 14 Suppl 1, pp.116–123.

Hamada, B.T. et al., 1998. Transendothelial Migration of Megakaryocytes in Response to Stromal Cell-derived Factor 1 (SDF-1) Enhances Platelet Formation. , 188(3).

Hashimoto, M. et al., 2008. S Phase-preferential Cre-recombination in mammalian cells revealed by HIV-TAT-PTD-mediated protein transduction. *Journal of biochemistry*, 143(1), pp.87–95.

He, N. et al., 2014. Bone marrow vascular niche: home for hematopoietic stem cells. *Bone marrow research*, 2014, p.128436.

Hellström, M. et al., 2007. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature*, 445(7129), pp.776–780.

Hooper, A.T. et al., 2009. Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2 mediated regeneration of sinusoidal endothelial cells. *Cell stem cell*, 4(3), pp.263–74.

Hu, J. et al., 2014. Endothelial Cell-Derived Angiopoietin-2 Controls Liver Regeneration as a Spatiotemporal Rheostat. *Science*, 343(January), pp.416–419.

Jakobsson, L. et al., 2010. Endothelial cells dynamically compete for the tip cell position during angiogenic sprouting. *Nature cell biology*, 12(10), pp.943–953.

Jin, F. et al., 2013. SDF-1 restores angiogenesis synergistically with VEGF upon LDL exposure despite CXCR4 internalization and degradation. *Cardiovascular research*, 100(3), pp.481–91.

Juarez, J. & Bendall, L., 2004. SDF-1 and CXCR4 in normal and malignant hematopoiesis. *Histology and Histopathology*, 19(1), pp.299–309.

Kawabata, K. et al., 1999. A cell-autonomous requirement for CXCR4 in long-term lymphoid and myeloid reconstitution. *Proceedings of the National Academy of Sciences of the United States of America*, 96(10), pp.5663–5667.

Kobayashi, H. et al., 2010. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. , 12(11), pp.1046–1056.

Koch, U. & Radtke, F., 2011. Notch in T-ALL: new players in a complex disease. *Trends in immunology*, 32(9), pp.434–42.

Koni, P.A. et al., 2001. Conditional vascular cell adhesion molecule 1 deletion in mice: impaired lymphocyte migration to bone marrow. *The Journal of experimental medicine*, 193(6), pp.741–54.

Kopp, H.G. et al., 2005. Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. *Blood*, 106(2), pp.505–513.

Kopp, H.-G. et al., 2005. The Bone Marrow Vascular Niche: Home of HSC Differentiation and Mobilization. *Physiology*, 20, pp.349–356.

Krebs, L.T. et al., 2004. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants service Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. , (207), pp.2469–2473.

Kubota, Y., Takubo, K. & Suda, T., 2008. Bone marrow long label-retaining cells reside in the sinusoidal hypoxic niche. *Biochemical and Biophysical Research Communications*, 366, pp.335–339.

Kunisaki, Y. et al., 2013. Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature*, 502(7473), pp.637–643.

De La Luz Sierra, M. et al., 2004. Differential processing of stromal-derived factor-1alpha and stromal-derived

factor-1beta explains functional diversity. *Blood*, 103(7), pp.2452–2459.

Lee, S.U. et al., 2013. LRF-mediated Dll4 repression in erythroblasts is necessary for hematopoietic stem cell maintenance. *Blood*, 121(6), pp.918–929.

Leuker, C.E. et al., 2001. Neonatally Induced Inactivation of the Vascular Cell Adhesion Molecule 1 Gene Impairs B Cell Localization and T Cell-Dependent Humoral Immune Response. *Journal of Experimental Medicine*, 193(6), pp.755–768.

Li, B. et al., 2010. Endothelial cells mediate the regeneration of hematopoietic stem cells. *Stem Cell Research*, 4(1), pp.17–24.

Li, X.M. et al., 2008. Bone marrow sinusoidal endothelial cells undergo nonapoptotic cell death and are replaced by proliferating sinusoidal cells in situ to maintain the vascular niche following lethal irradiation. *Experimental Hematology*, 36(9), pp.1143–1156.

Liu, Z.-J. et al., 2003. Regulation of Notch1 and Dll4 by Vascular Endothelial Growth Factor in Arterial Endothelial Cells : Implications for Modulating Arteriogenesis and Angiogenesis. *Society*, 23(1), pp.14–25.

Lobov, I.B. et al., 2007. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *PNAS*, 104(9), pp.1–6.

Lobov, I.B. et al., 2011. The Dll4/Notch pathway controls postangiogenic blood vessel remodeling and regression by modulating vasoconstriction and blood flow. *Blood*, 117(24), pp.6728–37.

Ma, Q. et al., 1998. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America*, 95(16), pp.9448–9453.

Ma, Q., Jones, D. & Springer, T.A., 1999. The Chemokine Receptor CXCR4 Is Required for the Retention of B Lineage and Granulocytic Precursors within the Bone Marrow Microenvironment. *Immunity*, 10, pp.463–471.

Marubini, F. et al., 1999. Dependence of Human Stem Cell Engraftment and Repopulation of NOD / SCID Mice on CXCR4. , 283(February), pp.845–848.

McNiece, I.K., Langley, K.E. & Zsebo, K.M., 1991. The role of recombinant stem cell factor in early B cell development. Synergistic interaction with IL-7. *J Immunol*, 146(11), pp.3785–3790.

Mirandola, L. et al., 2013. Anti-Notch treatment prevents multiple myeloma cells localization to the bone marrow via the chemokine system CXCR4/SDF-1. *Leukemia*, 27(November 2012), pp.1558–66.

Monvoisin, A. et al., 2006. VE-cadherin-CreER ^{T2} transgenic mouse: A model for inducible recombination in the endothelium. *Developmental Dynamics*, 235(12), pp.3413–3422.

Murone, M., Carpenter, D. a & de Sauvage, F.J., 1998. Hematopoietic deficiencies in c-mpl and TPO knockout mice. *Stem cells*, 16(1), pp.1–6.

Nagasawa, T. et al., 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature*, 15(382), pp.635–8.

Nagasawa, T., 2006. Microenvironmental niches in the bone marrow required for B-cell development. *Nature Reviews Immunology*, 6, pp.107–116.

Nie, Y., Han, Y.-C. & Zou, Y.-R., 2008. CXCR4 is required for the quiescence of primitive hematopoietic cells. *The Journal of experimental medicine*, 205(4), pp.777–83.

Niessen, K. et al., 2008. Slug is a direct Notch target required for initiation of cardiac cushion cellularization. *Journal of Cell Biology*, 182(2), pp.315–325.

Niswander, L.M. et al., 2014. SDF-1 dynamically mediates megakaryocyte niche occupancy and

thrombopoiesis at steady state and following radiation injury. *Blood*, 124(2), pp.277–286.

Noguera-Troise, I. et al., 2006. Blockade of Dll4 inhibits tumour growth by promoting non-productive angiogenesis. *Nature*, 444.

Nombela-Arrieta, C. et al., 2013. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nature cell biology*, 15(5), pp.533–43.

Phng, L.K. & Gerhardt, H., 2009. Angiogenesis: A Team Effort Coordinated by Notch. *Developmental Cell*, 16.

Pober, J.S. & Sessa, W.C., 2007. Evolving functions of endothelial cells in inflammation. *Nature Reviews Immunology*, 7(10), pp.803–815.

Poirault-Chassac, S. et al., 2010. Notch/Delta4 signaling inhibits human megakaryocytic terminal differentiation. *Blood*, 116(25), pp.5670–5678.

Poulos, M.G. et al., 2013. Endothelial Jagged-1 is necessary for homeostatic and regenerative hematopoiesis. *Cell Reports*, 4(5), pp.1022–1034.

Remédio, L. et al., 2012. Context- and cell-dependent effects of Delta-like 4 targeting in the bone marrow microenvironment. *PloS one*, 7(12), p.e52450.

Ribatti, D. & Crivellato, E., 2012. “Sprouting angiogenesis”, a reappraisal. *Developmental Biology*, 372(2), pp.157–165.

Roca, C. & Adams, R.H., 2007. Regulation of vascular morphogenesis by Notch signaling. *Genes and Development*, 21(20), pp.2511–2524.

Salter, A.B. et al., 2009. Endothelial progenitor cell infusion induces hematopoietic stem cell reconstitution in vivo. *Blood*, 113(9), pp.2104–2107.

Salvucci, O. et al., 2004. Evidence for the involvement of SDF-1 and CXCR4 in the disruption of endothelial cell-branching morphogenesis and angiogenesis by TNF-alpha and IFN-gamma. *Journal of leukocyte biology*, 76(1), pp.217–26.

Salvucci, O. et al., 2002. Regulation of endothelial cell branching morphogenesis by endogenous chemokine stromal-derived factor-1. *Blood*, 99(8), pp.2703–11.

de Sauvage, F.J. et al., 1996. Physiological regulation of early and late stages of megakaryocytopoiesis by thrombopoietin. *J Exp Med*, 183(2), pp.651–656.

Sekine, C. et al., 2012. Differential regulation of osteoclastogenesis by Notch2 / Delta-like 1 and Notch1 / Jagged1 axes. *Arthritis Research & Therapy*, 14(2), p.R45.

Shawber, C.J. & Kitajewski, J., 2004. Notch function in the vasculature: Insights from zebrafish, mouse and man. *BioEssays*, 26(3), pp.225–234.

Shutter, J.R., 2000. Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev.*, 14, pp.1313–1318.

Suchting, S. et al., 2007. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proceedings of the National Academy of Sciences of the United States of America*, 104(9), pp.3225–3230.

Sunamura, M. & Yagita, H., 2008. Neutralizing monoclonal antibody against human dll4.

Trindade, A. et al., 2012. Low-dosage inhibition of Dll4 signaling promotes wound healing by inducing functional neo-angiogenesis. *PloS one*, 7(1), p.e29863.

Trindade, A. et al., 2008. Overexpression of delta-like 4 induces arterialization and attenuates vessel formation in developing mouse embryos. *Blood*, 112(5), pp.1720–9.

Villa, N. et al., 2001. Vascular expression of Notch pathway receptors and ligands is restricted to arterial

vessels. *Mechanisms of Development*, 108(1–2), pp.161–164.

Williams, C.K. et al., 2008. Regulation of CXCR4 by the notch ligand delta-like 4 in endothelial cells. *Cancer Research*, 68(6), pp.1889–1895.

Williams, C.K. et al., 2006. Up-regulation of the Notch ligand Delta-like 4 inhibits VEGF-induced endothelial cell function. *Blood*, 107(3), pp.931–939.

Zeng, L. et al., 2012. Infusion of Endothelial Progenitor Cells Accelerates Hematopoietic and Immune Reconstitution, and Ameliorates the Graft-Versus-Host Disease After Hematopoietic Stem Cell Transplantation. *Cell Biochemistry and Biophysics*, 64(3), pp.213–222.

Zou, Y.R. et al., 1998. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature*, 393(6685), pp.595–599.

5.

DISCUSSION

CONTENTS

Not so long ago in 1966 endothelial cells lining blood vessels were described as a “layer of nucleated cellophane” and considered to be an inert barrier, working only as passive conduits for blood delivery (Florey 1966). Less than 60 years later, when the work included in this dissertation began, the view of endothelial cells had dramatically changed, and they were starting to be seen as instructive players in a multitude of processes through the release of paracrine factors, in a perfusion-independent manner (Butler, Kobayashi, et al. 2010; Ding et al. 2010; Ding et al. 2011; Kobayashi et al. 2010; Hooper et al. 2009; Butler, Nolan, et al. 2010; Ding et al. 2014). These endothelial-derived factors, collectively defined as angiocrine factors, include secreted and membrane-bound inhibitory and stimulatory growth factors, chemokines, cytokines, ECM components, exosomes and other cellular products that are produced by endothelial cells to help maintain homeostasis but that also orchestrate organ regeneration processes and tumor growth (Butler, Kobayashi, et al. 2010; Ding et al. 2010; Ding et al. 2011; Kobayashi et al. 2010; Hooper et al. 2009; Butler, Nolan, et al. 2010; Nolan et al. 2014; Rafii et al. 2016). Therefore, the cross-talk between endothelial cells and cells from the surrounding tissues has become a major focus in recent years, as it may lead to important findings of clinical relevance.

In this Thesis, we used genetically engineered mouse models to modulate the endothelial-specific expression of two ligands of the Notch signaling pathway and to characterize its consequences in two distinct processes: tumor progression and bone marrow recovery. Although both processes are markedly distinct from one another, they both rely on the expression of angiocrine factors and require the participation of hematopoietic cells. Therefore, we explored how the expression of Notch ligands influenced the interactions between endothelial and immune cells in both settings.

First, we explored the function of endothelial Jagged1 in the recruitment of macrophages into developing prostate tumors. This was achieved by targeting VE-Cadherin-positive vessels for conditional Jag1 knockout and Tie2-positive endothelial cells for conditional Jag1 overexpression, in TRAMP*Jag1$^{lox/lox}$*VE-Cadherin-Cre-ERT2 and TRAMP*Tet-O-Jag1*Tie2-rtTA mice, respectively. Then, we modulated the BM vascular niche by conditionally knocking out or overexpressing Dll4 in endothelial cells. A vast number of studies had suggested an involvement of Dll4 in vascular development and in hematopoietic cell-fate determination (Suchting et al. 2007; Hellström et al. 2007; Lobov

et al. 2007; Lobov et al. 2011; Hozumi et al. 2008; Lee et al. 2013; Poirault-Chassac et al. 2010), but its role in the BM vascular niche and in the cross talk of endothelial cells and hematopoietic cells in the BM was unknown.

5.1. THE ROLE OF ENDOTHELIAL JAGGED 1 IN MACROPHAGE RECRUITMENT IN A PROSTATE TUMOR MOUSE MODEL

The Notch pathway has become increasingly attractive as a therapeutic target for cancer. Jagged 1, particularly, is overexpressed in many cancer types and plays an important role in several aspects of tumor biology, such as tumor angiogenesis, tumor cell proliferation, epithelial-to-mesenchymal transition (EMT), metastasis and resistance to chemotherapy (Dai et al. 2014; Leong et al. 2007; Steg et al. 2011; Li et al. 2014; Reedijk et al. 2005; Zhang et al. 2006). Although the role of Jag1 in prostate cancer development has been studied (Zhang et al. 2006; Reedijk et al. 2005; Terada et al. 2014; Yu et al. 2014), the role of endothelial Jag1 (eJag1) in the progression of prostate cancer has only recently been addressed (A.-R. Pedrosa et al. 2015). In their study, Pedrosa and co-workers demonstrated that eJag1 not only has a pro-angiogenic effect, increasing tumor vascular density, maturation and perfusion, but also stimulates tumor cell proliferation and induces EMT. Although these effects were thought to derive solely from endothelial cell-cell or endothelial-tumor cell interactions, the authors failed to unravel whether eJag1 modulation could affect immune cell recruitment and activation, thereby promoting angiogenesis and tumor growth through distinct mechanisms.

During tumor progression, circulating monocytes and macrophages are attracted to the inflammatory signature in the tumor site, where they can rapidly differentiate into mature tumor-associated macrophages. Depending on the stimuli released by tumor and stromal cells, macrophages may acquire a M1 or M2 phenotype, the latter being known to induce angiogenesis and neoplastic cell growth (Chanmee et al. 2014; Sica & Mantovani 2012; Mantovani et al. 2002). Thus, we hypothesized that eJag1 was promoting macrophage polarization towards an M2 state, and therefore the phenotypes described by Pedrosa et al. upon eJag1 modulation could be caused also by an indirect effect of endothelial cells on other tumor stromal cells.

Interestingly, our results indicate that eJag1 not only increased the percentage of macrophages recruited to the tumor, it also shifted the ratio of M1 versus M2 macrophages towards an M2 state. The interactions established between endothelial cells and macrophages have been receiving a lot of attention in the last decade (Baer et al. 2013; Kubota et al. 2009; Squadrito & De Palma 2011; Mazzieri et al. 2011; Grunewald et al. 2006; Venneri et al. 2007; He et al. 2012; Tammela et al. 2011; Fantin et al. 2010). Nonetheless, the vast majority of reports focused on macrophage-induced angiogenesis, particularly in a tumor setting, and the role of endothelial cells on macrophage recruitment and polarization has only received minor attention.

When we started this project, endothelial cells had already been proposed as an instructive niche for functional polarization of macrophages toward an M2-like phenotype (He et al. 2012). The authors claimed that direct contact with the endothelium was necessary and sufficient for the induction of macrophage colonies and M2-like polarization, consistent with the observations that endothelial cells and macrophages engage in tight cell-cell contacts during developmental angiogenesis (Fantin et al. 2010) and in different tumor types (Mazzieri et al. 2011; Lewis & Pollard 2006; Lin et al. 2006; Squadrito & De Palma 2011; Lewis et al. 2007; De Palma et al. 2005), but the molecular players involved in macrophage adhesion and polarization remained unknown.

We found that endothelial Jag1 expression increased M2 polarization, both *in vivo* and *in vitro*. Although Notch signaling activation in macrophages had been described to induce M1 versus M2 polarization (Wang et al. 2010; Zhao et al. 2016), Notch receptor-ligand interactions can also have adhesion functions independently of Notch activation (Murata et al. 2012), so we thought that the increased differentiation into a pro-tumoral M2 phenotype could be caused by the increased Jag1:Notch adhesion or by eJag1-induced modulation of other angiocrine factors. Our preliminary results show that eJag1 overexpression induces an increase in *Dll4* and *Jag2* mRNA levels. Simultaneously, we observed that M2 macrophages present 4 to 7-fold higher levels of *Notch1* expression, when compared to M1 macrophages. This suggests that macrophages may be adhering to endothelial cells presenting either Jag1, Jag2 or Dll4 through their Notch1 receptor, and differentiating into an M2 phenotype in a Notch signaling independent-manner.

Nevertheless, our data also suggest angiocrine gene modulation, particularly in eJag1^{OE} mice, with *Cxcl12* and *Vcam1* downregulation and increased *Angpt2* expression. Although SDF1 and VCAM1 are involved in monocyte recruitment and adhesion to endothelial cells (Beider et al. 2014; Weber et al. 2007), respectively, they do not seem to be required for the recruitment or polarization of macrophages in eJag1^{OE} mice. *Angpt2* overexpression in eJag1^{OE} mice however could account for the higher numbers of macrophages in the tumor site, regardless of the downregulation in *Cxcl12* and *Vcam1*. Anpt2 promotes chemotaxis and invasion of Tie2-expressing monocytes/macrophages (TEMs) *in vitro* (Murdoch et al. 2007; Venneri et al. 2007), and its blockade hampers macrophage association with tumor blood vessels (Mazzieri et al. 2011). Consistently, overexpression of Angpt2 in the tumor vasculature increased the number of tumor TEMs and simultaneously enhanced their pro-angiogenic activity (Coffelt et al. 2010). Therefore, we believe that in our mouse model of prostate adenocarcinoma, endothelial-derived Angpt2 is one of the molecular players mediating macrophage recruitment and polarization in eJag1^{OE} mice. A more extensive analysis would be required to understand the mechanism through which Angpt2 exerts its function and to identify other endothelial-derived factors that are able to instruct macrophage differentiation, particularly in eJag1^{KO} mice.

Together, the results presented in **Chapter 2** provide additional information regarding the participation of endothelial Jag1 in solid tumor development. In addition to its direct role in angiogenesis and tumor growth, we show that endothelial Jag1 is likely contributing to tumor progression through the recruitment of macrophages into the tumor site and their polarization into a pro-angiogenic, pro-tumoral M2 state. In particular, we believe that this occurs through eJag1-mediated regulation of angiocrine gene expression, particularly *Dll4*, *Jagged2* and *Angpt2*, which in turn modifies the gene expression pattern of the tumor-associated macrophages (**Figure 5.1**).

It is interesting to note that in addition to endothelial cells, subsets of circulating monocytes and TAMs, as well as pericyte progenitors, all express the Angpt2 receptor, Tie2 (De Palma et al. 2005; De Palma et al. 2003; Venneri et al. 2007; Pucci et al. 2009; Welford et al. 2011). This observation may difficult the interpretation of our data because our gain-

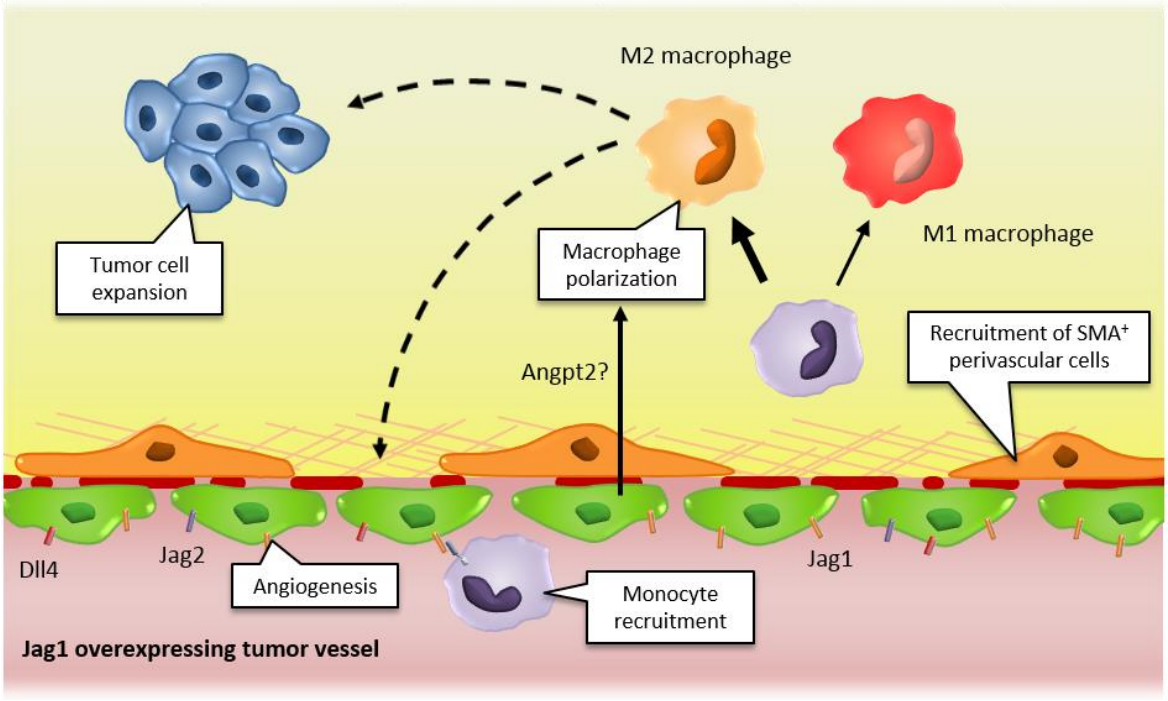

Figure 5.1. Our proposed model for the role of endothelial Jag1 in prostate tumor progression

Endothelial Jag1 promotes prostate tumor growth by directly inducing tumor cell expansion, angiogenesis and recruitment of SMA$^+$ perivascular cells. Furthermore eJag1 induces monocyte recruitment to the tumor site and their differentiation into M2 macrophages, which have a pro-tumoral function by indirectly (dashed arrows) promoting angiogenesis and neoplastic cell growth.

-of-function model overexpressed Jag1 in Tie2$^+$ cells, which also include the tumor-associated macrophages we were studying. Although the role of Jag1 expression in macrophage polarization is not clear, it is possible that the modulation of macrophage gene expression is a consequence of Jag1 upregulation in macrophages rather than an endothelial-derived effect. Interestingly, Jag1 is overexpressed following LPS or TNFα exposure (Morga et al. 2009; Goh et al. 2009; Johnston et al. 2009), suggesting that Jag1 expressing macrophages are polarized towards an M1 state, which may indicate that our data demonstrating macrophage M2 polarization is a result of Jag1 modulation in endothelial cells. Nonetheless, expression of Jag1 in other tumor stromal cells may increase the pro-angiogenic and pro-neoplastic effect observed in these mice. The use of an endothelial-specific promotor such as VE-Cadherin would be required to address whether the effects of Jag1 overexpression are exclusively endothelial-derived or if other stromal cells are also contributing for the increased angiogenesis, tumor growth and macrophage recruitment and polarization observed in these mice.

Although a number of questions remain to be answered, including the exact mechanism through which eJag1 regulates macrophage polarization, the findings argue in favor of Jag1-targeted therapies for prostate cancer. These therapies may be more promising than pan-Notch inhibitors, which induce gastrointestinal toxicity (Imbimbo 2008), or anti-Dll4 therapies, which have a low therapeutic window due to Dll4 haploinsufficiency (Gale et al. 2004; Duarte et al. 2004; Krebs et al. 2004) and only have direct effects on the tumor vasculature. Jag1, instead, has roles in a variety of cell types within the tumor, posing itself as a more attractive target for cancer therapies.

In addition, given the high similarity between endocrine tumors, this therapeutic approach may also be highly effective in treating breast and ovarian cancers, where Jag1 is often overexpressed (Choi et al. 2008; Haughian et al. 2012). This hypothesis is particularly supported by a study showing that targeting this Notch ligand in both tumor and stroma cells has synergistic effects in a model of ovarian cancer (Steg et al. 2011).

5.2. THE IMPACT OF ENDOTHELIAL DELTA-LIKE 4 MODULATION IN THE BM MICROENVIRONMENT AND HEMATOPOIESIS

The concept of hematopoietic stem cell (HSC) niche was first proposed almost 40 years ago by Schofield, who postulated that stem cells reside in a functionally and spatially characterized “niche” where they are prevented from differentiating and are sustained as stem cells (Schofield 1978). Although the osteoblastic niche was the first to be reported (Calvi et al. 2003; J. Zhang et al. 2003), the discovery of SLAM antigens enabled the histological assessment of HSC localization, leading to the discovery of the BM vascular niche (Kiel et al. 2005).

Over the last decade, the constantly evolving technological and experimental approaches have markedly improved our knowledge of the nature and function of the HSC niche. As noted in **Chapter 1**, the existence of an osteoblastic niche that directly contributes to HSC maintenance is now being questioned (Kiel et al. 2007; Kiel et al. 2009; Boulais & Frenette 2015) and distinct perivascular niches that differently regulate HSC fate have been

proposed (Boulais & Frenette 2015; Kunisaki et al. 2013). This hypothesis is consistent with recent observations revealing that the BM vascular niche is not composed of a uniform population of cells lining the blood vessels. Instead, the BM endothelial cells are remarkably heterogeneous, differing both in the vessel type and on the expression of specific factors (Wang et al. 2013; Remédio et al. 2012; Winkler et al. 2012; Kusumbe et al. 2014; Himburg et al. 2012), which is likely to create different microenvironments with distinct abilities to support HSCs or even specific stages of hematopoiesis. Thus, we reasoned that modulation of Dll4 in specific subsets of BM endothelial cells would modify these microenvironments, and shape the BM hematopoietic compartment accordingly.

A study performed in our lab in the beginning of this work had shown that, contrary to what was found in solid tumors, Dll4 blockade did not induce endothelial cell proliferation or enhanced sprouting and branching in the BM, but instead shifted endothelial cell identity (Remédio et al. 2012). However, this study failed to address whether the hematopoietic changes observed in anti-Dll4 treated mice were a direct effect of Dll4 blockade on endothelial cells. Although Dll4 was shown to be largely restricted to blood vessels in the BM microenvironment (**Figure 5.2**) (Lee et al. 2013), other cells, such as mature osteoblasts, were also found to express Dll4 (Yu et al. 2015). Our hypothesis was that modulation of endothelial Dll4 (eDll4) would induce changes in both the BM vascular niche and in the hematopoietic lineages by modifying the angiocrine gene expression pattern of the targeted endothelial cells.

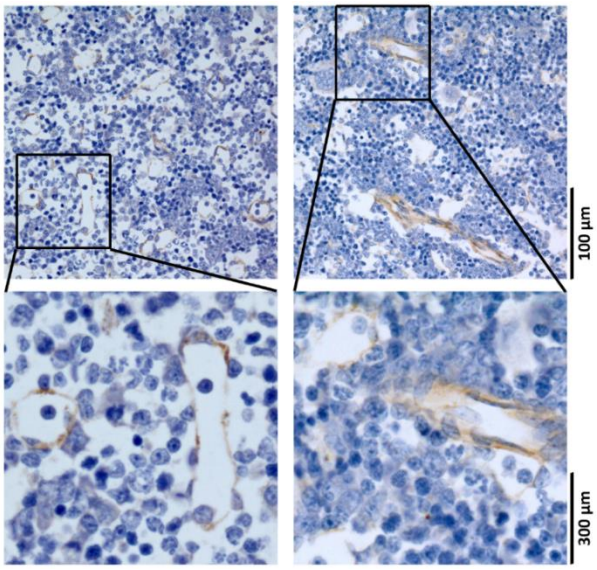

Figure 5.2. Delta-like 4 expression in the bone marrow

Representative immunohistochemistry images of femur bone marrows stained with Dll4 (R&D AF1389) and counterstained with hematoxylin, showing that Dll4 expression in the BM is mainly restricted to capillary and sinusoidal endothelial cells. Insets are magnified 3x.

Similarly to what Remédio described, we found that knocking out or overexpressing Dll4 specifically in endothelial cells resulted in changes in BM vessel identity following myeloablation, without increasing the overall BM vessel content. The endothelial markers that varied with eDll4 modulation, VEGFR2 and VE-Cadherin, were found to be expressed in BM sinusoidal cells that stimulate HSPC self-renewal through expression of specific angiocrine genes (Hooper et al. 2009; Butler, Nolan, et al. 2010; Kobayashi et al. 2010; Wang et al. 2013) and to be involved in the mobilization of BM hematopoietic cells.

Interestingly, although the number of both VE-Cadherin^{+} and VEGFR2^{+} vessels increased in eDll4^{KO} mice, they have opposite effects: whereas VE-Cadherin negatively mediates HSPC transendothelial migration (van Buul et al. 2002), VEGFR2 is required for vascular dilation and mobilization of BM-derived cells to tumors and peripheral tissues (Lim et al. 2014). This suggests retention of HSPCs in the BM and mobilization of differentiated hematopoietic cells as a possible mechanism that contributes to accelerate eDll4^{KO} mice recovery.

As discussed in **Chapter 4**, this modulation in BM vessel identity was only observed following myeloablation and not at steady-state possibly because the effects of eDll4 modulation in the BM vascular structures was only visible after severely damaging the BM sinusoids (by sub-lethally irradiating mice) (H. G. Kopp et al. 2005; Hooper et al. 2009; Li et al. 2008; Li et al. 2010) and allowing the surviving endothelial cells to proliferate and replace the injured cells (Li et al. 2008). Interestingly, although VE-Cadherin and VEGFR2 have opposing effects in hematopoietic cell mobilization, they exert a synergistic effect in maintaining VEGF-mediated endothelial cell survival (Carmeliet et al. 1999), which suggests that eDll4 knockdown may be favorable for the recovery of the BM vascular niche following myeloablation.

The reconstitution of the BM vascular niche is essential for hematopoietic recovery (H. G. Kopp et al. 2005; Hooper et al. 2009; Li et al. 2010; Salter et al. 2009; Chute et al. 2007). Conditional deletion of VEGFR2 blocks the regeneration of BM sinusoids in irradiated mice, preventing hematopoietic reconstitution and HSPC engraftment (Hooper et al. 2009). Furthermore, deletion of pro-apoptotic proteins in BM endothelial cells is enough to protect HSCs from radiation injury and induce 100% survival in lethally irradiated mice (Doan et al. 2013). A similar phenotype was observed upon transplantation of endothelial

cells or EPCs following lethal doses of irradiation (Chute et al. 2007; Li et al. 2010; Salter et al. 2009), even though a minority of these cells was incorporated into the BM vasculature (Slayton et al. 2007), suggesting that healthy endothelial cells are required for both maintenance of the BM vasculature and hematopoietic reconstitution, most likely through the expression of a specific set of angiocrine factors. Therefore, a rapid regeneration of the BM sinusoidal endothelial cells in mice bearing lower eDll4 levels (eDll4^{KO} and Control OE) is probably inducing a faster hematopoietic recovery, as observed by the increased recovery in BM cellularity in sub-lethally irradiated mice and the reduced BM damage detected in lethally irradiated Control KO mice that received a total BM transplantation from eDll4^{KO} mice. This suggests that the changes in vascular identity observed following eDll4 modulation create specialized niches that distinctly support hematopoiesis through differential angiocrine gene expression.

In **Chapter 3**, we first hypothesized that erythropoiesis and myelopoiesis were regulated by distinct vessels in the BM. However, the observations that eDll4 knockout also affects megakaryocyte numbers, possibly by decreasing their migration towards the sinusoids, implies that eDll4 downregulation in sinusoids affects both erythrocyte and megakaryocyte lineages. Nonetheless, although eDll4 knockdown decreases MK number, it must be inducing platelet production, as we do not see any differences in the hemograms of these animals. In addition, the opposite is likely to be occurring upon eDll4 overexpression, where the platelet numbers were decreased, even though MK numbers were similar to the Control OE mice. This is consistent with earlier reports showing that Dll4 enhances erythroid cell formation (Dando et al. 2005; Laranjeiro et al. 2012) and decreases platelet production by reducing mature megakaryocytes (Poirault-Chassac et al. 2010), and suggests that Dll4-derived modulation of the hematopoietic lineages is dependent on Notch signaling activation on the hematopoietic cells, in a microenvironment independent-manner.

Our findings that B cells are more likely to be attached to vessels with higher levels of Dll4 is consistent with a role of the Notch ligands as adhesion molecules, as suggested above. As discussed in **Chapter 4**, the proximity of B cells to the BM sinusoids is likely to be inducing their mobilization towards the periphery. Furthermore, although the osteoblasts have been suggested to create a niche for certain early lymphoid progenitors (Wu et al.

2008; Zhu et al. 2007; Greenbaum et al. 2013), the contact of lymphoid progenitors with Dll4 expressing vessels is likely to activate the Notch signaling pathway, leading to decreased levels of B cells. However, these effects are somehow delayed, since eDll4 modulation of both B cell migration and B cell percentage in the BM are only observed in later stages of BM recovery, even though BM vessel identity and recruitment of B cells towards the base of BM sinusoids is observed 8 days following exposure to radiation.

As stated in **Chapter 3**, mice with lower levels of eDll4 have higher variations in the percentages of myeloid and lymphoid compartments by day 8 after irradiation but, by day 26 of recovery, they also reach relative proportions of the hematopoietic lineages that resemble more closely their non-irradiated counterparts. This might indicate that eDll4 knockout induces rapid BM recovery following myeloablation first by inducing the regeneration of the BM vascular niche and only afterwards through hematopoietic reconstitution. Thus, we provide experimental evidences that implicate endothelial Dll4 as potential target for the development of novel therapeutic strategies for patients subjected to chemotherapy or BM transplants.

Indeed, although the management of acute myelosuppression caused by chemotherapy or radiation therapy given to cancer patients has improved significantly in recent years with the use of various hematopoietic growth factors (Wang et al. 2006), Dll4 inhibitors should be investigated has a means to reduce the abrasive effects of such therapies on the hematopoietic system.

In addition, Dll4 has emerged as a promising target to prevent tumor angiogenesis, and a number of Dll4 inhibitors (MEDI0639, enoticumab, demcizumab, and OMP-305B83) (Jenkins et al. 2012; Chiorean et al. 2015; Smith et al. 2014; Yen et al. 2016) are currently being tested in Phase 1 and Phase 2 clinical trials for a variety of solid tumors. This suggests that anti-Dll4 therapies may be used in combination with chemotherapy not only to promote tumor regression, but also to prevent chemotherapy-derived myelosuppression, the main adverse effect of chemotherapy regimens, exerting a dual role in cancer patients.

In summary, we show that loss of endothelial Dll4 in sub-lethally irradiated mice induces changes in the vascular identity and angiocrine gene expression that improve the recovery of the BM vascular niche and hematopoietic reconstitution, albeit higher levels of

B cells and platelet production and decreased erythropoiesis were observed (**Figure 5.3**). Endothelial Dll4 knockout in donor BM in a BM transplant setting may also reduce BM damage and enhance hematopoietic recovery.

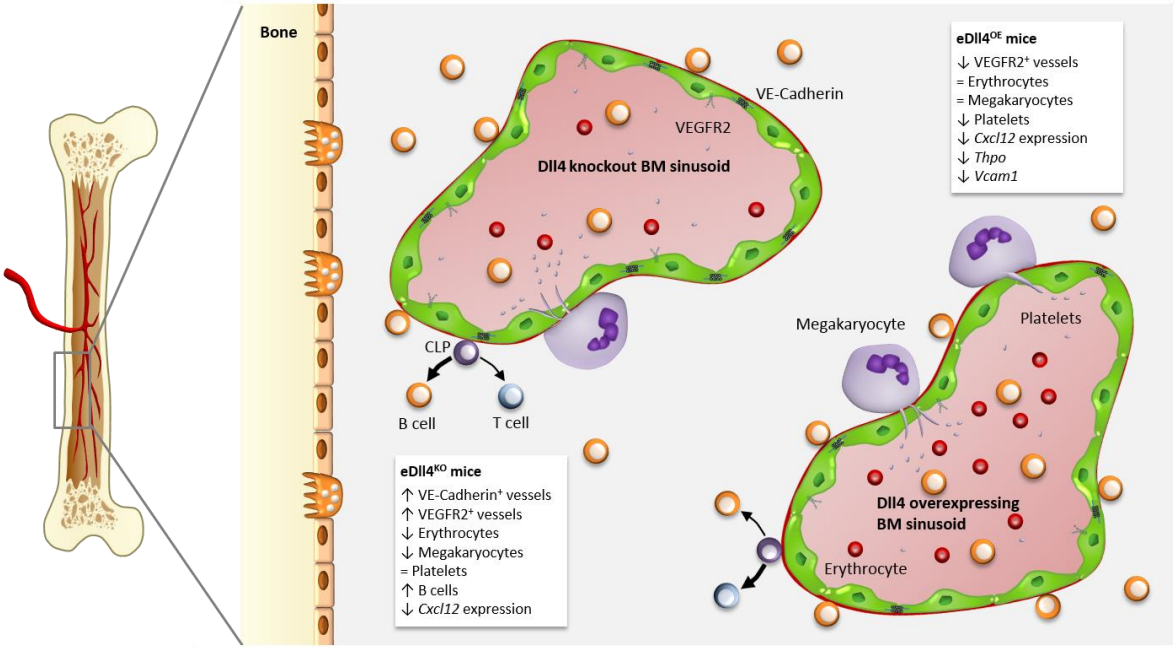

Figure 5.3. Major findings included in Chapters 3 and 4

Schematic model of the BM microenvironment in eDll4^{KO} and eDll4^{OE} mice. Our data suggests that following myeloablation, the number of BM vessels does no change, but the identity of BM sinusoid is modified, with eDll4^{KO} mice presenting increased number of VEGFR2^{+} and VE-Cadherin^{+} vessels, which gives them an advantage in the recovery of the BM vascular niche. eDll4^{OE} mice had decreased VEGFR2^{+} vessels. Hematopoietic reconstitution was also affected by eDll4 modulation: eDll4^{KO} mice had an increased percentage of B cells in the BM, but fewer cells in close contact with the vessel wall, resulting in reduced migration, possibly due to the downregulation of *Cxcl12*. eDll4^{KO} mice also presented reduced erythrocyte numbers, but eDll4 overexpression did not affect the erythroid lineage. Megakaryocyte numbers were reduced in eDll4^{KO} mice, but the platelet number were similar to Control KO, whereas in eDll4^{OE}, MK number was similar to Control OE, but the platelet numbers were reduced, possibly due to a downregulation in *Thpo* and *Cxcl12*.

5.3. CONCLUDING REMARKS

In this Thesis, we provide relevant data on the function of endothelial-specific expression of Notch ligands in prostate tumor progression and in BM reconstitution following myelosuppression, which may be useful for the development of potential therapies directed against the Notch signaling pathway.

Over the past 6 years, endothelial cells have emerged as instructive players in distinct physiological and pathological tasks, sustaining the homeostasis of resident stem cells, orchestrating the regeneration and repair of adult organs and inducing tumor growth, through the release of distinct angiocrine factors (Butler, Kobayashi, et al. 2010; Ding et al. 2010; Ding et al. 2011; Kobayashi et al. 2010; Hooper et al. 2009; Butler, Nolan, et al. 2010; Nolan et al. 2014; Ding et al. 2014). The microvascular bed of each organ is composed of specialized endothelial cells that are programmed to satisfy the angiocrine function and metabolic demands of that particular organ (Nolan et al. 2014). Disruption of stem-cell homeostasis and impairment of organ repair (without compromising blood supply) through deletion of specific angiocrine factors have helped uncover the tissue-specific instructive function of endothelial cells (Ding et al. 2014; Ding et al. 2012; Ding et al. 2010; Ding & Morrison 2013; Butler, Nolan, et al. 2010; Himburg et al. 2012; Winkler et al. 2012; Ding et al. 2011; Hooper et al. 2009; Doan et al. 2013).

Specifically, although Jag1 had already been shown to be an essential angiocrine factor in prostate tumor progression, through induction of neoplastic cell growth, angiogenesis and vessel maturation (A.-R. Pedrosa et al. 2015), we have demonstrated that it may also be indirectly inducing tumor growth by recruiting and polarizing tumor-associated macrophages into a pro-tumoral state. Consistently, endothelial Jag1 knockout markedly inhibited tumor growth (A.-R. Pedrosa et al. 2015), indicating that Jag1 may be an interesting therapeutic target in prostate cancer patients.

We have also explored the BM microenvironment and hematopoietic changes in sub-lethally irradiated mice upon eDll4 modulation. Our data suggests that endothelial Dll4 impairs the rapid recovery of the BM vascular niche which results in delayed hematopoietic reconstitution. Furthermore, depletion of eDll4 in donor mice decreases BM damage and enhances the recovery of BM cellularity following BM transplants, indicating that Dll4 is an angiocrine factor that should be explored as a therapeutic target in patients receiving chemotherapy or in BM transplants.

5.4. REFERENCES

Baer, C. et al., 2013. Reciprocal interactions between endothelial cells and macrophages in angiogenic vascular niches. *Experimental Cell Research*, 319(11), pp.1626–1634.

Beider, K. et al., 2014. Multiple myeloma cells recruit tumor-supportive macrophages through the CXCR4/CXCL12 axis and promote their polarization toward the M2 phenotype. *Oncotarget*, 5(22), pp.11283–96.

Boulais, P.E. & Frenette, P.S., 2015. Making sense of hematopoietic stem cell niches. *Blood*, 125(17), pp.2621–9.

Butler, J.M., Nolan, D.J., et al., 2010. Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell*, 6(3), pp.251–264.

Butler, J.M., Kobayashi, H., et al., 2010. Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. *Nature reviews. Cancer*, 10(2), pp.138–146.

van Buul, J.D. et al., 2002. Migration of human hematopoietic progenitor cells across bone marrow endothelium is regulated by vascular endothelial cadherin. *Journal of Immunology*, 168(0022–1767 (Print)), pp.588–596.

Calvi, L.M. et al., 2003. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*, 425(6960), pp.841–846.

Carmeliet, P. et al., 1999. Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell*, 98(2), pp.147–57.

Chanmee, T. et al., 2014. Tumor-associated macrophages as major players in the tumor microenvironment. *Cancers*, 6(3), pp.1670–1690.

Chiorean, E.G. et al., 2015. A phase I first-in-human study of enoticumab (REGN421), a fully human delta-like ligand 4 (Dll4) monoclonal antibody in patients with advanced solid tumors. *Clinical Cancer Research*, 21(12), pp.2695–2703.

Choi, J.H. et al., 2008. Jagged-1 and Notch3 juxtacrine loop regulates ovarian tumor growth and adhesion. *Cancer Research*, 68(14), pp.5716–5723.

Chute, J.P. et al., 2007. Transplantation of vascular endothelial cells mediates the hematopoietic recovery and survival of lethally irradiated mice. *Blood*, 109(6), pp.2365–72.

Coffelt, S.B. et al., 2010. Angiopoietin-2 regulates gene expression in TIE2-expressing monocytes and augments their inherent proangiogenic functions. *Cancer Research*, 70(13), pp.5270–5280.

Dai, Y. et al., 2014. Silencing of Jagged1 inhibits cell growth and invasion in colorectal cancer. *Cell death & disease*, 5(4), p.e1170.

Dando, J.S. et al., 2005. Notch/Delta4 interaction in human embryonic liver CD34+ CD38- cells: positive influence on BFU-E production and LTC-IC potential maintenance. *Stem cells*, 23(4), pp.550–560.

Ding, B.-S. et al., 2014. Divergent angiocrine signals from vascular niche balance liver regeneration and fibrosis. *Nature*, 505(7481), pp.97–102.

Ding, B.-S. et al., 2011. Endothelial-Derived Angiocrine Signals Induce and Sustain Regenerative Lung Alveolarization. *Cell*, 147(3), pp.539–553.

Ding, B.-S. et al., 2010. Inductive angiocrine signals from sinusoidal endothelium are required for liver regeneration. *Nature*, 468(7321), pp.310–315.

Ding, L. et al., 2012. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature*, 481(7382), pp.457–62.

Ding, L. & Morrison, S.J., 2013. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature*, 495(7440), pp.231–5.

Doan, P.L. et al., 2013. Tie2(+) bone marrow endothelial cells regulate hematopoietic stem cell regeneration following radiation injury. *Stem cells (Dayton, Ohio)*, 31(2), pp.327–37.

Duarte, A. et al., 2004. Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes and Development*, 18(20), pp.2474–2478.

Fantin, A. et al., 2010. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. *Blood*, 116(5), pp.829–840.

Florey, Lord, 1966. The Endothelial Cell. *British Medical Journal*, 2(5512), pp.487–490.

Gale, N.W. et al., 2004. Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proceedings of the National Academy of Sciences of the United States of America*, 101(45), pp.15949–15954.

Goh, F. et al., 2009. Selective induction of the Notch ligand Jagged-1 in macrophages by soluble egg antigen from Schistosoma mansoni involves ERK signalling. *Immunology*, 127(3), pp.326–337.

Greenbaum, A. et al., 2013. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature*, 495(7440), pp.227–30.

Grunewald, M. et al., 2006. VEGF-induced adult neovascularization: Recruitment, retention, and role of accessory cells. *Cell*, 124(1), pp.175–189.

Haughian, J.M. et al., 2012. Maintenance of hormone responsiveness in luminal breast cancers by suppression of Notch. *Proceedings of the National Academy of Sciences of the United States of America*, 109(8), pp.2742–2747.

He, H. et al., 2012. Endothelial cells provide an instructive niche for the differentiation and functional polarization of M2-like macrophages. *Blood*, 120(15), pp.3152–3162.

Hellström, M. et al., 2007. Dll4 signalling through Notch1 regulates formation of tip cells during angiogenesis. *Nature*, 445(7129), pp.776–780.

Himburg, H.A. et al., 2012. Pleiotrophin regulates the retention and self-renewal of hematopoietic stem cells in the bone marrow vascular niche Heather. *Cell Reports*, 2(4), pp.946–975.

Hooper, A.T. et al., 2009. Engraftment and reconstitution of hematopoiesis is dependent on VEGFR2 mediated regeneration of sinusoidal endothelial cells. *Cell stem cell*, 4(3), pp.263–74.

Hozumi, K. et al., 2008. Delta-like 4 is indispensable in thymic environment specific for T cell development. *The Journal of experimental medicine*, 205(11), pp.2507–2513.

Imbimbo, B.P., 2008. Therapeutic potential of gamma-secretase inhibitors and modulators. *Current topics in medicinal chemistry*, 8(1), pp.54–61.

Jenkins, D.W. et al., 2012. MEDI0639: A Novel Therapeutic Antibody Targeting Dll4 Modulates Endothelial Cell Function and Angiogenesis In Vivo. *Molecular Cancer Therapeutics*, 11(8), pp.1650–1660.

Johnston, D.A., Dong, B. & Hughes, C.C.W., 2009. TNF induction of jagged-1 in endothelial cells is NFκB-dependent. *Gene*, 435(1.2), pp.36–44.

Kiel, M.J. et al., 2009. Hematopoietic Stem Cells Do Not Depend on N-Cadherin to Regulate Their Maintenance. *Cell Stem Cell*, 4(2), pp.170–179.

Kiel, M.J. et al., 2005. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*, 121(7), pp.1109–21.

Kiel, M.J., Radice, G.L. & Morrison, S.J., 2007. Lack of Evidence that Hematopoietic Stem Cells Depend on N-Cadherin-Mediated Adhesion to Osteoblasts for Their Maintenance. *Cell Stem Cell*, 1(2), pp.204–217.

Kobayashi, H. et al., 2010. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. , 12(11), pp.1046–1056.

Kopp, H.G. et al., 2005. Tie2 activation contributes to hemangiogenic regeneration after myelosuppression. *Blood*, 106(2), pp.505–513.

Krebs, L.T. et al., 2004. Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants service Haploinsufficient lethality and formation of arteriovenous malformations in Notch pathway mutants. , (207), pp.2469–2473.

Kubota, Y. et al., 2009. M-CSF inhibition selectively targets pathological angiogenesis and lymphangiogenesis. *The Journal of experimental medicine*, 206(5), pp.1089–1102.

Kunisaki, Y. et al., 2013. Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature*, 502(7473), pp.637–643.

Kusumbe, A.P., Ramasamy, S.K. & Adams, R.H., 2014. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. *Nature*, 507(7492), pp.323–8.

Laranjeiro, R. et al., 2012. The notch ligand delta-like 4 regulates multiple stages of early hemato-vascular development. *PLoS ONE*, 7(4).

Lee, S.U. et al., 2013. LRF-mediated Dll4 repression in erythroblasts is necessary for hematopoietic stem cell maintenance. *Blood*, 121(6), pp.918–929.

Leong, K.G. et al., 2007. Jagged1-mediated Notch activation induces epithelial-to-mesenchymal transition through Slug-induced repression of E-cadherin. *The Journal of experimental medicine*, 204(12), pp.2935–2948.

Lewis, C.E., De Palma, M. & Naldini, L., 2007. Tie2-expressing monocytes and tumor angiogenesis: Regulation by hypoxia and angiopoietin-2. *Cancer Research*, 67(18), pp.8429–8432.

Lewis, C.E. & Pollard, J.W., 2006. Distinct role of macrophages in different tumor microenvironments. *Cancer Research*, 66(2), pp.605–612.

Li, B. et al., 2010. Endothelial cells mediate the regeneration of hematopoietic stem cells. *Stem Cell Research*, 4(1), pp.17–24.

Li, D. et al., 2014. The notch ligand JAGGED1 as a target for anti-tumor therapy. *Frontiers in oncology*, 4(September), p.254.

Li, X.M. et al., 2008. Bone marrow sinusoidal endothelial cells undergo nonapoptotic cell death and are replaced by proliferating sinusoidal cells in situ to maintain the vascular niche following lethal irradiation. *Experimental Hematology*, 36(9), pp.1143–1156.

Lim, S. et al., 2014. VEGFR2-mediated vascular dilation as a mechanism of VEGF-induced anemia and bone marrow cell mobilization. *Cell Reports*, 9(2), pp.569–580.

Lin, E.Y. et al., 2006. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Research*, 66(23), pp.11238–11246.

Lobov, I.B. et al., 2007. Delta-like ligand 4 (Dll4) is induced by VEGF as a negative regulator of angiogenic sprouting. *PNAS*, 104(9), pp.1–6.

Lobov, I.B. et al., 2011. The Dll4/Notch pathway controls postangiogenic blood vessel remodeling and regression by modulating vasoconstriction and blood flow. *Blood*, 117(24), pp.6728–37.

Mantovani, A. et al., 2002. Macrophage polarization: Tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in Immunology*, 23(11), pp.549–555.

Mazzieri, R. et al., 2011. Targeting the ANG2/TIE2 Axis Inhibits Tumor Growth and Metastasis by Impairing Angiogenesis and Disabling Rebounds of Proangiogenic Myeloid Cells. *Cancer Cell*, 19(4), pp.512–526.

Morga, E. et al., 2009. Jagged1 regulates the activation of astrocytes via modulation of NFκB and JAK/STAT/SOCS pathways. *Glia*, 57(16), pp.1741–1753.

Murata, A. et al., 2012. A Notch Ligand, Delta-Like 1 Functions As an Adhesion Molecule for Mast Cells.

Murdoch, C. et al., 2007. Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. *Journal of immunology (Baltimore, Md. : 1950)*, 178(11), pp.7405–7411.

Nolan, D.J. et al., 2014. Molecular Signatures of Tissue-Specific Microvascular Endothelial Cell Heterogeneity in Organ Maintenance and Regeneration. *Developmental Cell*, 26(2).

De Palma, M. et al., 2003. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. *Nature medicine*, 9(6), pp.789–95.

De Palma, M. et al., 2005. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell*, 8(3), pp.211–226.

Pedrosa, A.-R. et al., 2015. Endothelial Jagged1 promotes solid tumor growth through both pro-angiogenic and angiocrine functions. *Oncotarget*, 6(27).

Poirault-Chassac, S. et al., 2010. Notch/Delta4 signaling inhibits human megakaryocytic terminal differentiation. *Blood*, 116(25), pp.5670–5678.

Pucci, F. et al., 2009. A distinguishing gene signature shared by tumor-infiltrating Tie2-expressing monocytes, blood "resident" monocytes, and embryonic macrophages suggests common functions and developmental relationships. *Blood*, 114(4), pp.901–914.

Rafii, S., Butler, J.M. & Ding, B.-S., 2016. Angiocrine functions of organ-specific endothelial cells. *Nature*, 529(7586), pp.316–325.

Reedijk, M. et al., 2005. High-level coexpression of JAG1 and NOTCH1 is observed in human breast cancer and is associated with poor overall survival. *Cancer Research*, 65(18), pp.8530–8537.

Remédio, L. et al., 2012. Context- and cell-dependent effects of Delta-like 4 targeting in the bone marrow microenvironment. *PloS one*, 7(12), p.e52450.

Salter, A.B. et al., 2009. Endothelial progenitor cell infusion induces hematopoietic stem cell reconstitution in vivo. *Blood*, 113(9), pp.2104–2107.

Schofield, R., 1978. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood cells*, 4(1–2), pp.7–25.

Sica, A. & Mantovani, A., 2012. Macrophage plasticity and polarization: in vivo veritas. *The Journal of clinical investigation*, 122(3), pp.787–795.

Slayton, W.B. et al., 2007. The role of the donor in the repair of the marrow vascular niche following hematopoietic stem cell transplant. *Stem cells (Dayton, Ohio)*, 25(11), pp.2945–55.

Smith, D.C. et al., 2014. A phase I dose escalation and expansion study of the anticancer stem cell agent demcizumab (Anti-DLL4) in patients with previously treated solid tumors. *Clinical Cancer Research*, 20(24), pp.6295–6303.

Squadrito, M.L. & De Palma, M., 2011. Macrophage regulation of tumor angiogenesis: Implications for cancer therapy. *Molecular Aspects of Medicine*, 32(2), pp.123–145.

Steg, A.D. et al., 2011. Targeting the Notch ligand jagged1 in both tumor cells and stroma in ovarian cancer. *Clinical Cancer Research*, 17(17), pp.5674–5685.

Suchting, S. et al., 2007. The Notch ligand Delta-like 4 negatively regulates endothelial tip cell formation and vessel branching. *Proceedings of the National Academy of Sciences of the United States of America*, 104(9), pp.3225–3230.

Tammela, T. et al., 2011. VEGFR-3 controls tip to stalk conversion at vessel fusion sites by reinforcing Notch signalling. *Nature Cell Biology*, 13(10), pp.1202–1213.

Terada, N. et al., 2014. Correlation of Sprouty1 and Jagged1 With Aggressive Prostate Cancer Cells With Different Sensitivities to Androgen Deprivation. *J Biol Chem.*, 115(9), pp.1505–1515.

Venneri, M.A. et al., 2007. Identification of proangiogenic TIE2-expressing monocytes (TEMs) in human peripheral blood and cancer. *Blood*, 109(12), pp.5276–5285.

Wang, L. et al., 2013. Identification of a clonally expanding haematopoietic compartment in bone marrow. *The EMBO journal*, 32(2), pp.219–30.

Wang, Y., Probin, V. & Zhou, D., 2006. Cancer therapy-induced residual bone marrow injury-Mechanisms of induction and implication for therapy. *Current Cancer Therapy Reviews*, 2(3), pp.271–9.

Wang, Y.C. et al., 2010. Notch signaling determines the M1 versus M2 polarization of macrophages in antitumor immune responses. *Cancer Research*, 70(12), pp.4840–4849.

Weber, C., Fraemohs, L. & Dejana, E., 2007. The role of junctional adhesion molecules in vascular inflammation. *Nature Reviews Immunology*, 7(6), pp.467–477.

Welford, A.F. et al., 2011. TIE2-expressing macrophages limit the therapeutic efficacy of the vascular-disrupting agent combretastatin A4 phosphate in mice. *Journal of Clinical Investigation*, 121(5), pp.1969–1973.

Winkler, I.G. et al., 2012. Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nature Medicine*, 18(11), pp.1651–1657.

Wu, J.Y. et al., 2008. Osteoblastic regulation of B lymphopoiesis is mediated by Gs{alpha}-dependent signaling pathways. *Proceedings of the National Academy of Sciences of the United States of America*, 105(44), pp.16976–81.

Yen, W.-C. et al., 2016. Abstract C164: Dual targeting of the DLL4 and VEGF pathways with a bispecific monoclonal antibody inhibits tumor growth and reduces cancer stem cell frequency. *Molecular Cancer Therapeutics*, 14(12 Supplement 2).

Yu, V.W.C. et al., 2015. Specific bone cells produce DLL4 to generate thymus-seeding progenitors from bone marrow. *Journal of Experimental Medicine*, 212(5), pp.759–74.

Yu, Y. et al., 2014. Androgen receptor promotes the oncogenic function of overexpressed Jagged1 in prostate cancer by enhancing cyclin B1 expression via Akt phosphorylation. *Mol Cancer Res*, 12(6), pp.830–842.

Zhang, J. et al., 2003. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*, 425, pp.836–841.

Zhang, Y. et al., 2006. Down-regulation of Jagged-1 induces cell growth inhibition and S phase arrest in prostate cancer cells. *International journal of cancer. Journal international du cancer*, 119(9), pp.2071–7.

Zhao, J.-L. et al., 2016. Forced Activation of Notch in Macrophages Represses Tumor Growth by Upregulating miR-125a and Disabling Tumor-Associated Macrophages. *Cancer Research*.

Zhu, J. et al., 2007. Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. *Blood*, 109(9), pp.3706–12.

Annex

THE IMPACT OF CHRONIC INTERMITTENT HYPOXIA ON HEMATOPOIESIS AND THE BONE MARROW MICROENVIRONMENT

Alvarez-Martins, I. et al., 2016. The impact of chronic intermittent hypoxia on hematopoiesis and the bone marrow microenvironment. *Pflügers Archiv - European Journal of Physiology*.

Pflugers Arch - Eur J Physiol
DOI 10.1007/s00424-016-1797-6

SIGNALING AND CELL PHYSIOLOGY

The impact of chronic intermittent hypoxia on hematopoiesis and the bone marrow microenvironment

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Received: 9 November 2015 / Revised: 21 December 2015 / Accepted: 26 January 2016

Abstract Obstructive sleep apnea (OSA) is a highly prevalent sleep-related breathing disorder which is associated with patient morbidity and an elevated risk of developing hypertension and cardiovascular diseases. There is ample evidence for the involvement of bone marrow (BM) cells in the pathophysiology of cardiovascular diseases but a connection between OSA and modulation of the BM microenvironment had not been established. Here, we studied how chronic intermittent hypoxia (CIH) affected hematopoiesis and the BM microenvironment, in a rat model of OSA. We show that CIH followed by normoxia increases the bone marrow hypoxic area, increases the number of multipotent hematopoietic progenitors (CFU assay), promotes erythropoiesis, and increases monocyte counts. In the BM microenvironment of CIH-subjected animals, the number of VE-cadherin-expressing blood vessels, particularly sinusoids, increased, accompanied by increased smooth muscle cell coverage, while vWF-positive vessels decreased. Molecularly, we investigated the expression of endothelial cell-derived genes (angiocrine factors) that could explain the cellular phenotypes. Accordingly, we observed an increase in colony-stimulating factor 1, vascular endothelium growth factor, delta-like 4, and angiopoietin-1 expression. Our data shows that CIH induces vascular remodeling in the BM microenvironment, which modulates hematopoiesis, increasing erythropoiesis, and circulating monocytes. Our study reveals for the first time the effect of CIH in hematopoiesis and suggests that hematopoietic changes may occur in OSA patients.

Keywords Chronic intermittent hypoxia · Hematopoiesis · Vascular niche · Bone marrow microenvironment

Introduction

Hematopoiesis has long been known to be affected by environmental hypoxia [64]. Despite numerous reports relating hypoxia with hematopoietic modulation [3, 5, 44, 46, 48, 49, 61, 64] and the great attention currently given to hypoxia-inducible factor (HIF) [34], the current scientific knowledge largely relies on of studies performed under acute hypoxia stimulation in isolated systems. Therefore, the role of chronic systemic hypoxia in the bone marrow (BM) microenvironment and hematopoiesis is still unknown.

Here, we studied the role of environmental hypoxia using a clinically relevant chronic intermittent hypoxia (CIH) model, which consists of exposing the experimental animals to a paradigm of CIH for 30–35 days, as a model of obstructive sleep apnea syndrome (OSA) [25]. OSA is an increasingly prevalent condition affecting children and adults, which is renowned as a frequent secondary cause of hypertension [16]. There is ample evidence for the involvement of BM-derived

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Electronic supplementary material The online version of this article (doi:10.1007/s00424-016-1797-6) contains supplementary material, which is available to authorized users.

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Published online: 09 February 2016

cells in the pathophysiology of hypertension [78] but a relation between OSA and modulation of the BM microenvironment had not been shown and is the subject of the present study. The clinical hematological aspects of OSA are still largely unknown, with several studies reporting mainly platelet activation and increased hematocrit, but not assessing BM or circulating blood cell alterations [22]. Furthermore, these studies typically compared groups of patients with differing disease phenotypes [32] or treated versus untreated patients [42] but not healthy controls versus patients and do not explore the mechanisms involved [22].

Mechanistically, we focused on the vascular compartment of the BM, because it mediates the differentiation and proliferation of hematopoietic cells, as well as their egress from the BM microenvironment [40]. Furthermore, one of the most striking effects of hypoxia is the promotion of angiogenesis, which results from hypoxia inducible factor (HIF)-mediated increase in vascular endothelial growth factor (VEGF) expression [62].

The causal relationship between intermittent hypoxia and increased VEGF expression is known in both OSA patients [43] and healthy volunteers [7], but the relevance of this finding is still unknown.

The present work aims to explore for the first time whether CIH induce changes in the BM vascular compartment, which might in turn modulate hematopoiesis.

Our data suggests that CIH may promote erythropoiesis, increase the multipotential progenitor cell-derived CFUs accompanied by an increase in BM myeloid and B lymphocyte counts and a decrease BM T cells. Additionally, CIH also induces expansion of the blood monocyte compartment and perturbs the BM microenvironment by interfering with the vascular niche. Together, our results reveal hematopoietic and hematological complications of CIH which need to be validated and evaluated in a clinical setting.

Methods

Animals

Experiments were performed in twelve (12) male Wistar rats, aged 8–12 weeks, obtained from the NOVA Medical School animal facility. Animals were housed in polycarbonate cages, under 12-h light/dark cycles (8 am–8 pm) at a room temperature 22 ± 2.0 °C and relative humidity 60 ± 10 %. Rats were maintained one or two per cage with ad libitum access to food and water. Applicable institutional and governmental regulations concerning ethical use of animals were followed, according to the NIH Principles of Laboratory Animal Care (NIH Publication 85–23, revised 1985), the European guidelines for the protection of animals used for scientific purposes (European Union Directive 2010/63/EU), and the Portuguese Law n° 113/2013. Experimental procedures were previously approved (nr. 21/2013/CEFCM) by the Institutional Ethics Committee of the NOVA Medical School for animal care and use in research.

In vivo experiments

Rats were divided into two groups: normoxia and CIH. Animals were kept in a eucapnic atmosphere, inside of medium A-chambers (76 × 51 × 51 cm, A-60274-P, Biospherix Ltd, NY, USA) with ad libitum food and water access. The chambers were equipped with gas injectors and sensors for oxygen (O_2) and carbon dioxide (CO_2) levels in order to ensure the accuracy of CIH cycles. Accumulation of CO_2 was prevented by the continuous flow of the gas mixtures through vent holes and the presence in the chamber of self-indicating soda lime, which absorbs the expired CO_2. The CO_2 levels inside the chambers never exceeded 1 %. A silica gel container was also placed inside the chambers in order to absorb water. Oxygen concentration inside the chambers was controlled using 100 % nitrogen (N_2) and 100 % O_2 by an electronically regulated solenoid switches in a three-channel gas mixer, which gradually lowered oxygen in the chamber from 21 to 5 % O_2 (OxyCycler AT series, Biospherix Ltd, NY, USA). The chambers were infused with 100 % N_2 for 3.5 min to briefly reduce the O_2 concentration to 5 % and then with 100 % O_2 for 7 min to restore oxygen to ambient levels of 21 %, until the start of the next CIH cycle. Each CIH cycle lasted 10.5 min, and rats were exposed during their sleep period (light phase of light/dark cycle) to 5.6 CIH cycles/h, 10.5 h/day for 32 days and analyzed 3 days after the hypoxic period. During the remaining hours of the day, the chambers were ventilated with a constant flow of room air to keep oxygen levels at 21 %. O_2 was purchased as regular gas bottles (Gasin, Portugal), while N_2 was generated from the air by pressure swing adsorption technology using a high-output nitrogen generator (Nitrogen 15 Plus, PSA Technology, Sysadvance, Maia, Portugal).

Sample collection

After exposing rats to 32 days of hypoxia followed by 3 days in normoxia, rats were sacrificed by intraperitoneal injection with medetomidine (0.5 mg/kg body weight, Domitor®, Pfizer Animal Health) and ketamine (75 mg/kg body weight, Imalgene 1000®, Mérial, Lyon, France), and cardiac puncture was performed to collect peripheral blood. Blood was collected in EDTA-coated tubes (Multivette 600, Sarstedt), and plasma sampling and complete blood counts were performed. Femur BM cells were flushed out with PBS 2 % FBS, and the total BM cell count was assessed using a Burker hemocytometer (Blau Brand).

Hypoxia quantification in bone marrow sections

Three days after the hypoxic period, the subjects were intravenously injected (via tail vein) with 60-mg/kg pimonidazole hydrochloride (Hypoxyprobe, Inc, Burlington, USA), a misonidazole-based compound, which forms adducts with thiol groups of proteins, peptides, and amino acids specifically in hypoxic cells ($pO_2 < 10$ mmHg) (81). Two hours later, rats were euthanized using 60-mg/kg sodium pentobarbital IV (Eutasil, Ceva Santé Animale, Libourne, France) and transcardially perfused with PBS. Quantification of BM hypoxic areas was performed using the ImageJ software in 10 high-power fields (×400 magnification) per animal.

Flow cytometry

Bone marrow and peripheral blood cells were treated with red blood cell lysis buffer (Biolegend) for 15 min in the dark and were then stained for anti-CD90 (HIS51) fluorescein isothiocyanate (FITC), anti-CD11b (WT.5) allophycocyanin (APC) (BD Biosciences), anti-CD117 (2B8) APC, anti-CD19 (1D3) PE-cyanine 7 (PE/Cy7) (eBiosciences), and anti-CD3 (17A2) APC-cyanine 7 (APC/Cy7) (BioLegend). Flow cytometric analyses were carried out using an LSR Fortessa flow cytometer equipped with FACS Diva 6.2 Software (BD Biosciences). Data were analyzed with a FlowJo 9.8.2 software.

In vitro colony forming assay

Femur BM cells were flushed with PBS 2 mM EDTA, treated with red blood cell lysis buffer (Biolegend) for 15 min in the dark and plated onto petri dishes for 2 h. Non-adherent cells (10^5 cells) were collected and plated onto a semi-solid cytokine-supplemented methylcellulose medium (MethoCult GF M3434) (Stemcell Technologies). Each colony formed in this semi-solid medium is single-cell derived and represents the identity of the original progenitor cell [6, 15]. The resulting colonies were scored after 1–2 weeks of culture, according to manufacturer's instructions.

Immunostaining and imaging

Femurs were formalin-fixed, decalcified with formic acid for 3 days, and processed for routine histopathology. Immunohistochemistry staining was performed on 3-μm slices. Sections were treated for antigen retrieval and incubated with the primary antibodies listed in Table 1 for 1 h at room temperature, immunostained according to the visualization system manufacturer's instructions and counterstained with hematoxylin. The slides were then analyzed using a Leica DM2500 microscope, and all images were acquired with the 40× objective. The number of vessels or cells stained by each marker was quantified as a mean of 10 representative images of individual rat femurs. Sections for immunofluorescence were incubated with VE-cadherin for 1 h at room temperature followed by an incubation with an Alexa Fluor 488 secondary antibody (Life Technologies). DNA was stained with DAPI Vectashield mounting medium (H-1200, Vector Laboratories). Imaging was performed using a Zeiss LSM 510 META microscope, and images were acquired with the 40× water immersion objective.

RNA isolation and quantitative PCR

Bone marrow cells were collected by flushing off tibias with PBS 2 % FBS. Cells were centrifuged at 1200 rpm for 5 min, collected to TRIzol Reagent (Invitrogen), and RNA was extracted according to manufacturer's instructions. Reverse transcription was performed with SuperScript II (Invitrogen), according to the manufacturer's protocol. Quantitative PCR was performed with Power SYBR Green PCR Master Mix (Roche) on a ViiA^{TM} 7 Real-Time PCR System (Life Technologies). The sequences of the oligonucleotides used are included in Table 2. A primer concentration of 180 nM was found to be optimal in all cases. Amplification of hypoxanthine guanine phosphoribosyl transferase (*Hprt*) was used for sample normalization.

Statistical analysis

Results are expressed as mean ± standard deviation. Data were analyzed using unpaired two-tailed student's *t* test. *P* values of <0.05 were considered statistically significant.

Results

Chronic intermittent hypoxia does not affect BM cell number but modulates/perturbs hematopoiesis

In this study, six male Wistar rats were exposed to chronic intermittent hypoxia for 32 days and then left in normoxia for three more days. The post-hypoxic period before the analysis allowed us to observe the persistent changes in hematopoiesis and the BM microenvironment. Notably, as assessed by hypoxyprobe staining, the extent of BM hypoxia was increased in CIH exposed animals (Fig. 1a, b), which was accompanied by an upregulation in *Hif1a* messenger RNA (mRNA) (Fig. 1c), CIH was also associated with a significant decrease in whole body weight (Fig. 1d), an observation that had already been associated with both sustained and intermittent hypoxia exposure [45, 76]. Nevertheless, concerning the BM cellular content (corrected to the total body weight of the animals), there was no alteration in the total number of BM cells caused by CIH (Fig. 1e). However, the percentage of

Table 1 Primary antibodies used for immunohistochemistry

Antigen	Antigen retrieval	Dilution	Brand
CD105 (Endoglin)	HIER, Tris-EDTA pH 9	1:150	R&D AF1320
CD11b	HIER, Tris-EDTA pH 9	1:100	BD 550282
SMA	HIER, Tris-EDTA pH 9	1:500	DAKO HHF35
VE-cadherin	PIER, Pepsin	1:150	R&D AF1002
vWF	PIER, Pepsin	1:300	DAKO A0082
Anti-goat, peroxidase		ready-to-use	VectorLabs MP-7405
Anti-mouse, peroxidase		ready-to-use	DAKO K4007
Anti-rabbit, peroxidase		ready-to-use	DAKO K4011
Anti-rat, peroxidase		ready-to-use	VectorLabs MP-7444

specific hematopoietic lineages within the BM changed. Specifically, we found an increase in the $CD11b^+$ myeloid cells (the majority of which are monocytes) in hypoxia-exposed animals (from 36.10±5.60 to 48.38±5.86 %), and a modulation in the lymphoid compartment, with a significant increase in $CD19^+$ B cells (18.07±4.55 % in normoxia and 28.90±5.40 % in CIH) and a decrease in the percentage of $CD3^+$ T lymphocytes (2.74±0.18 and 1.70±0.26 % in normoxia and CIH exposed rats, respectively) (Fig. 2b–d). Although we did not observe a significant variation in the percentage of $CD90^+$/c-kit^+ stem and progenitor cells by flow cytometry (Fig. 2a and a'), in vitro colony-forming units (CFU) assays revealed an increase in 1.5-fold in the total number of CFUs in animals from the CIH group (Fig. 2f). Such assays allowed us to identify and count single-cell derived colonies, representing either multipotent (CFU-granulocyte-erythrocyte-macrophage-megakaryocyte, CFU-GEMM) or monopotent (CFU-monocyte, CFU-M; CFU-granulocyte, CFU-G or bursting forming units-erythrocyte, BFU-E) progenitors. Our data show that chronic intermittent hypoxia treatment significantly increased CFU-M, CFU-G and BFU-E colony numbers, without significant alterations in the multipotent capacity (CFU-GEMM) of treated rats (Fig. 2g). Although these results suggest CIH favors the expansion of monopotent progenitors, we cannot rule out the possibility that the monopotent progenitors arose from more primitive multipotent progenitors.

Having characterized the changes in BM hematopoietic lineages accounted to CIH exposure, we asked whether these alterations could also be identified in peripheral blood (PB). Indeed, as shown in Fig. 3, we observed a significant increase in erythrocyte counts, in hemoglobin and in the hematocrit of CIH exposed rats (Fig. 3a). Our data also shows a decrease in circulating lymphocytes and an increase in monocytes upon CIH exposure (Fig. 3b), a finding that was also confirmed by flow cytometry, showing increased $CD11b^+$ myeloid cells in the PB (Fig. 2e). In contrast, leukocyte and granulocyte (eosinophil and neutrophil) counts were not affected by CIH. Platelet counts and mean platelet volume were also similar in the CIH and normoxia groups (Fig. 3c).

Our data thus mimic some of the clinical aspects observed in OSA patients, as we observed no changes in platelet counts, but fails to reproduce other symptoms, such as the increase in platelet activation and aggregation, as assessed by mean platelet volume. Moreover, we report for the first time an increase in the myeloid compartment, both the BM and PB and a modulation in the percentage of B and T lymphocytes in the BM of animals exposed to CIH.

Chronic intermittent hypoxia affects the bone marrow vasculature and modulates monocyte counts

Having shown that exposure of rats to CIH perturbs hematopoiesis, as evidenced by changes in circulating mononuclear cell and erythrocyte counts, next we sought to characterize the phenotypic and molecular alterations that occurred in the BM microenvironment that could account for such changes. As shown in Fig. 4, the BM vasculature of CIH exposed animals suffered "phenotypic alterations," as shown by the significant increase in VE-cadherin expressing vessels (Fig. 4c', c") and their VE-cadherin coverage (Fig. 4f), the increase in smooth muscle cell coverage (Fig. 4d', d"), and the decrease in the number of vessels that were positive for vWF (Fig. 4a', a"). In contrast, endoglin (CD105)-expressing vessels did not vary upon CIH exposure (Fig. 4b', b"). Similarly, the BM megakaryocyte content (also assessed by vWF staining) did not vary with CIH exposure (Fig. 4a', a"').

To demonstrate that there was undoubtedly an increased expansion of myeloid cells within the BM, we assessed the BM $CD11b^+$ cell (monocyte) content. In accordance with the flow cytometric and complete blood count data, we observed an increment in the number of BM monocytes in CIH treated animals (Fig. 4e', e").

Together, these data show the BM vasculature and in particular the VE-cadherin and vWF-expressing vessels, and the pericyte/smooth muscle cell vessel coverage are affected by CIH exposure. This morphological change in BM vessels of CIH-treated animals is accompanied by a significant increase in the number of CD11b + monocytes.

Table 2 List of primers used for RT-PCR

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
rHPRT	GACCGCTTTTCCCGCGAGCC	TCACGACGCTGGGACTGAGGG
rAdm	ACCGCACGGCTCGACACTTC	TCCCACGACTTAGCGCCCAC
rAngpt1	TGATGCCTGTGGCCCTTCCA	CATGGTTTTGCCCCGCAGTGT
rAngpt2	TGTCCGGCGAGGAGTCCAAC	GATTTTGCCCGCCGTGCCTG
rBmp4	AGTTTGTTCAAGATTGGCTCCC	CGACCATCAGCATTCGGTTA
rCdh2	TCTGCACCAGGTTTGGAATGGGT	ACATACGTCCCAGGCTTTGATCCC
rCsf1	GCCACCGAGAGGCTACAGGAA	TTTGGACACAGGCCTCGTTCTGTT
rCsf2	GGTCTACGGGGCAACCTCACC	AGTTTCCGGGGTTGGAGGGCA
rCsf3	CCTCGGGGTGGCCCCTACTG	CCCGACGCTGGAAGGCAGAA
rCxcl12	GCATCAGTGACGGTAAGCCA	TCTCAAAGAATCGGCAGGGG
rCxcr4	TGGAGAGCGAGCATTGCC	GCAGGGTTCCTTGTTGGAGT
rDhh	TCCCCAACTACAACCCCGA	GCTAGAGCATTCACCCGCTC
rDkk1	CTCTATGAGGGCGGGAACAA	GCAAGGGTAGGGCTGGTAGT
rDll1	TCTCCTGACGACCTCGCAACA	GGTGCCTCTGTGTGGTCAGGC
rDll4	CTGGCCGGGAACCTTCTCACTC	TCTCTGGCCGCAGGTCGTCTC
rEpo	CCCTATTTACGGGGTGCTGG	CTGTCTCTGCCCCTGAGTTC
rFgf1	AGGGACAGGAGCGACCAGCA	TACACTTCGCCCGCGCTTTCC
rFgf2	TCCGGGAGAAGAGCGACCCA	CCGGTTCGCACACACTCCCTTG
rFlt3l	AGCTCTGAAGCCCTGTATCGGGA	ACTGCACCTCCAGGCACCGA
rFlt4	CCCTGCTTGGTGTCCATTCC	GTCGTCCCACAACACCTCC
rHes1	TCAACACGACACCGGACAA	GCTTTGATGACTTTCTGTGCT
rHey1	GCCGACGAGACCGAATCAA	TTCGCAGATCCCTGCTTCTC
rHey2	CCCTTGCGAGGAGACGACCT	GCTCCCCACGTCGATGGTCT
rHif1α	GCTTACACACAGAAATGGCCC	GTCCTCCCCCGGCTTGTTAG
rHif2α	CCGCCTCATGTCTCCATGTT	CAGCTTGTTGGACAGGGCTA
rIgf1	CTTTGCGGGGCTGAGCTGGT	AGCCCCTTGGTCCACACACGAA
rIgfbp3	AAGGCGCTGCTGAATGGCCG	GCTGGGAGGGGAGGTAGGCA
rIgfbp5	ACCTGCCCAACTGTGACCGC	GGCCACGAGAAGGCTTGCACT
rIl3	TGATGCTCTTCCACCAGGGACT	AGTCCTGCAATCCAACGTCCTGA
rIl6	CTCTCCGCAAGAGACTTCCAGC	AGGGAAGGCAGTGGCTGTCAA
rIl11	CCGACTGGAACGGCTACTTC	CAAGGCTAGGCGAGACATCAAG
rJag1	GGAAGGCTGGATGGGTCCTGA	TGCAGGAGCCATGCTTGGGA
rJag2	CGGGCCTCGTCGTCATTCCCT	CAGGCCTCCACGATGAGGGTGA
rKdr	CGGTCATCCTCACCAATCCC	CCGATCTGGGGTGGAACATT
rKitl	ACAAAACTGGTGGCGAATCTTCCAA	TCCCGGCGACATAGTTGAGGGT
rPecam1	TGGCTTGAGTGGGCGGATGG	AGCCGGGTGGCTGAGGGAAG
rSmad2	TGTGCAGAGCCCCAACTGTAACCA	GGATTTTGCACACTGTCGCGGG
rSmad3	AGGCCATCACCACGCAGAACG	AGCCGGCCATCCAGTGACCT
rTgfb1	AGCCCGAGGCGGACTACTAC	TGCGTTGTTGCGGTCCACCATT
rThpo	TGTCCCCACCCCACTCTGTGC	GTGTGGGGCCTCTCCCCTGA
rVcam1	CGGAGCCTCAACGGTACTTTGG	GCGAGCGTTTTGTATTCAGGGGA
rVegfa	GCACTGGACCCTGGCTTTAC	TCTGCTCCCCTTCTGTCGT

Chronic intermittent hypoxia modulates the expression of "angiocrine genes"

Since CIH affected the BM vasculature as evidenced by the increased VE-cadherin-expressing vessels, next, we hypothesized the "vascular gene expression" could also be altered. Therefore, we assessed the expression of the so-called "angiocrine genes," which are expressed by the BM endothelial cells and have been previously shown to be essential for BM recovery following stresses such as irradiation or exposure to chemotherapy [9]. In detail, we sought for molecular correlates to the phenotypic changes observed in the BM

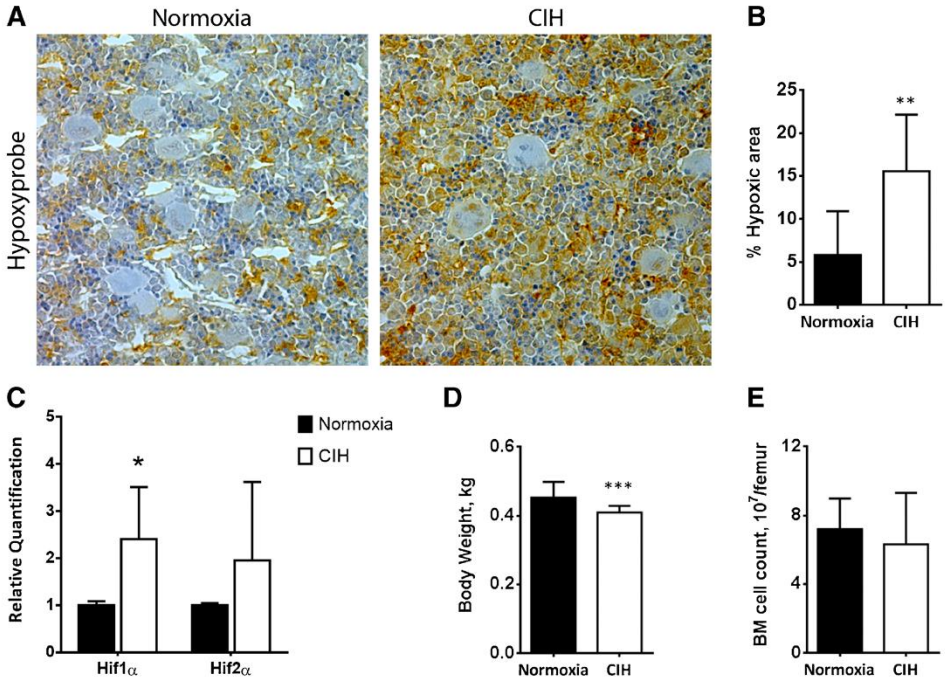


Fig. 1 Chronic intermittent hypoxia affects the hypoxic state of the bone marrow and decreases body weight but does not affect bone marrow cell counts. **a** The extent of BM hypoxia was increased in animals exposed to CIH, as assessed by pimonidazole staining and **b** the significant increase in hypoxic area in CIH animals. **c** CIH also have increased *Hif1α* expression. **d** Rats subjected to CIH had a lower body weight than controls. **e** Total BM cell count shows that CIH does not modify BM cellularity. Results are represented as the mean ± SD of bone marrow sections from six male Wistar rats (*$p < 0.05$; **$p < 0.01$)

microenvironment; that is, genes that could explain the increased erythropoiesis, monocytosis, and vascular changes in the BM microenvironment. As shown in Fig. 5, CIH increased the expression of colony-stimulating factor 1 (*Csf1*), previously shown to modulate monocyte differentiation, proliferation, and survival [63]. Moreover, *Vegfa*, delta-like 4 (*Dll4*), angiopoietin 1 (*Angpt1*) and Fms-like tyrosine kinase 4 (*Flt4*) also increased upon CIH exposure (Fig. 5), suggesting these may be involved in the vascular response observed in the BM of CIH-exposed animals, namely, the increase in VE-cadherin-expressing and SMA-covered BM vessels.

Discussion

Chronic intermittent hypoxia (CIH) was first reported by Fletcher and colleagues as a model for obstructive sleep apnea [25]. In their protocol, mice were exposed to cycles of intermittent hypoxia for 7 h each day for 35 days and were found to develop a long-term hypertensive response to CIH, as it is observed in OSA patients. Such model fails to reproduce the transient hypercapnia that is observed in OSA patients, determined by airway occlusion, and mimics only the intermittent hypoxic episodes that occur chronically in these patients, allowing us to separate the mechanical component of obstruction from the effect of intermittent hypoxia itself. In fact, the main goal of our work was to explore the effects of CIH by itself in the BM vascular compartment. Additionally, over the years, several other groups have shown that this rat model of CIH mimics many aspects of the obstructive sleep apnea syndrome, such as atherosclerosis and alterations in the hematological parameters (for a review see [16]), and only few have manipulated the CO_2 levels inside the chambers [19, 36, 58].

It remains unclear whether the partial pressure of CO_2 in the arterial blood ($PaCO_2$) is relevant in humans. Hypercapnia is not a standard parameter analyzed in polysomnographic recordings in patients and therefore there is no consensus on the impact of $PaCO_2$ in arterial blood pressure and other parameters in patients with OSA. For instance, in clinical studies of patients with moderate OSA, the changes in $PaCO_2$ have seemed to be irrelevant [24] or have shown a slight increase [74] during the apneic events. Combining chronic sustained hypoxia with hypercapnia was shown to restore the subcutaneous PaO_2 to levels close to the normoxic rats [72] and to

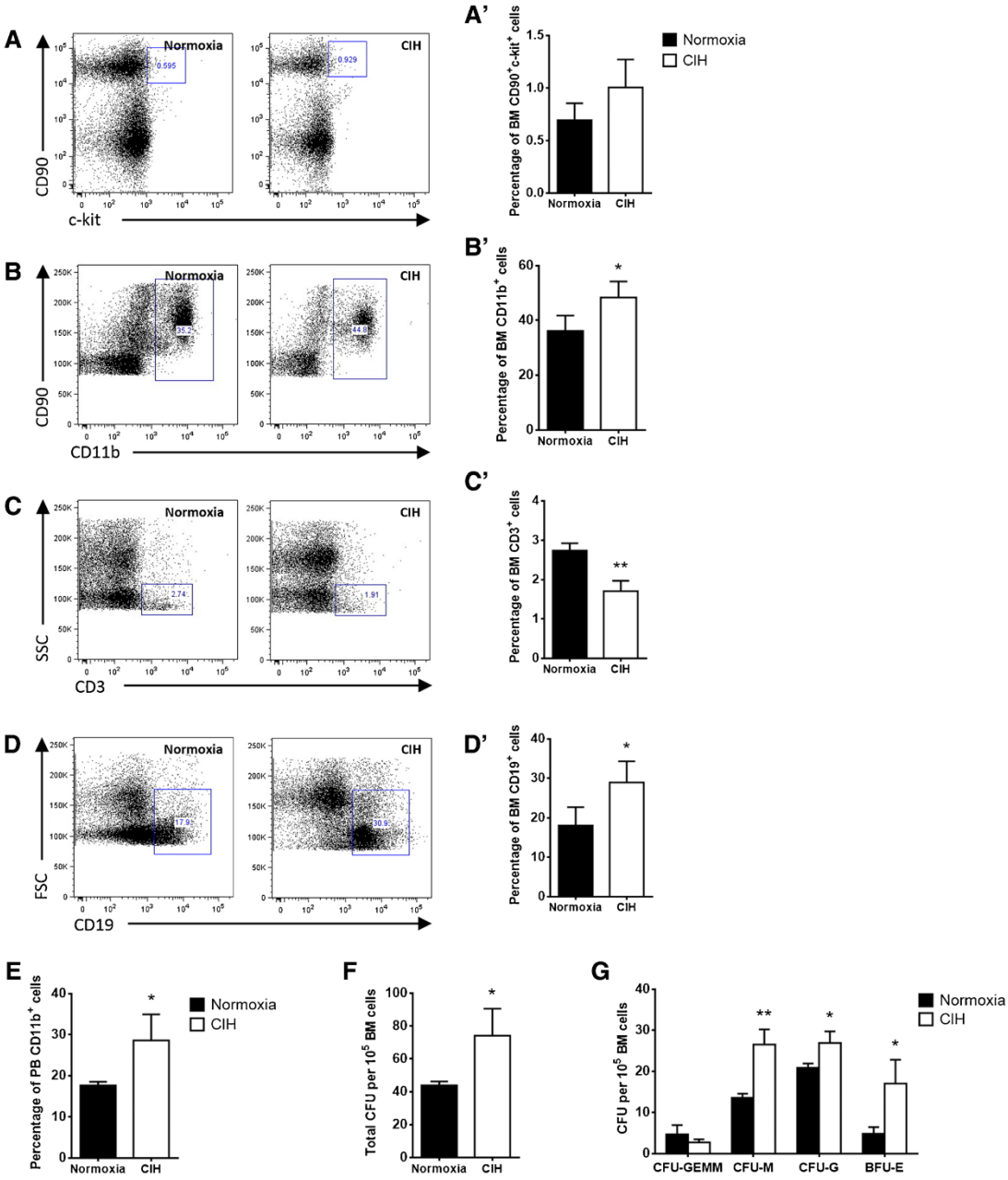


Fig. 2 Rats exposure to chronic intermittent hypoxia affects specific hematopoietic lineages and the commitment of bone marrow progenitor cells. **a**–**d** Representative plots of the flow cytometric analysis of bone marrow cells from normoxic ($n = 5$) and CIH ($n = 5$) exposed rats. Quantification of **a'** CD90+/c-kit + stem and progenitor cells did not reveal a significant alteration in CIH animals. However, **b'** $CD11b^+$ myeloid- and **d'** $CD19^+$ B cells were increased as opposed to **(c')** CD3 T lymphocytes that decreased upon CIH exposure. **e** Quantification of peripheral blood $CD11b^+$ cells by flow cytometry also revealed an increase in the percentage of those cells in circulation. **f** Colony-forming unit counts from methylcellulose culture of 10^5 BM cells reveal that CIH treatment induces an increased the number of HSPC, **g** with a particular increase in macrophage, granulocyte and erythroid (CFU-M, CFU-G and BFU-E) colonies. Results are represented as the mean ± SD of bone marrow cells from five male Wistar rats (*$p < 0.05$; **$p < 0.01$)

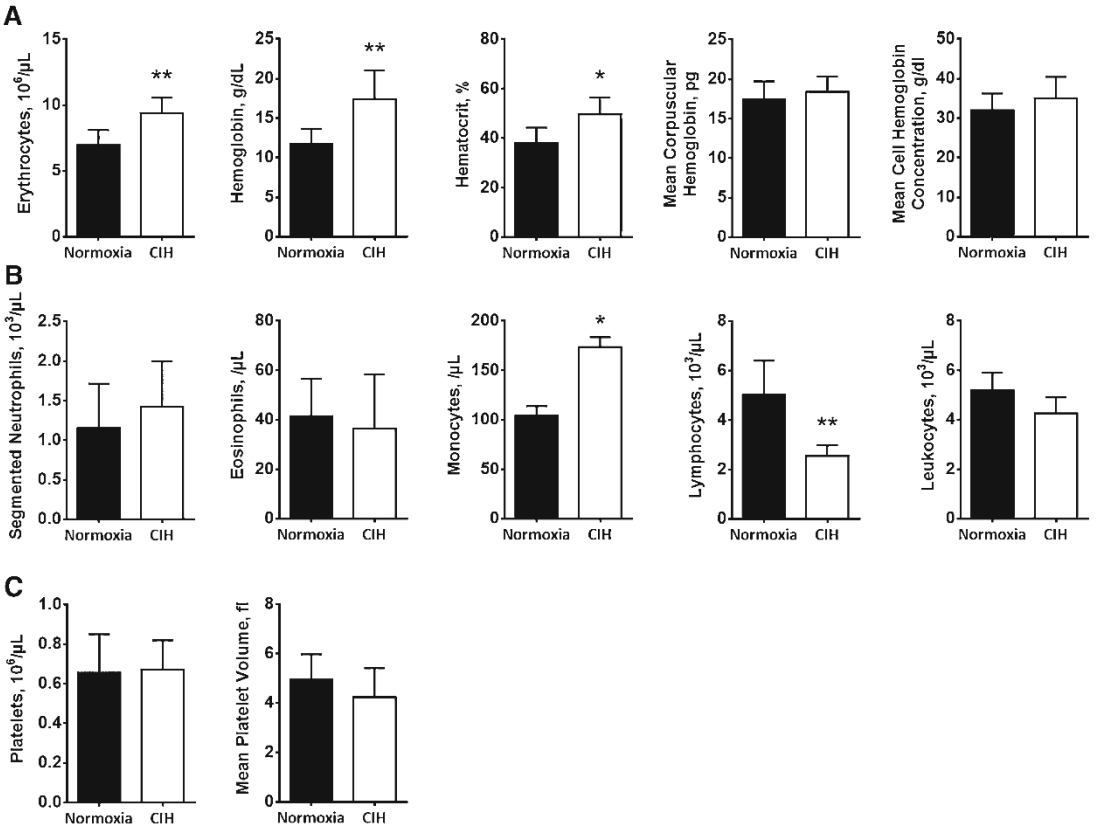
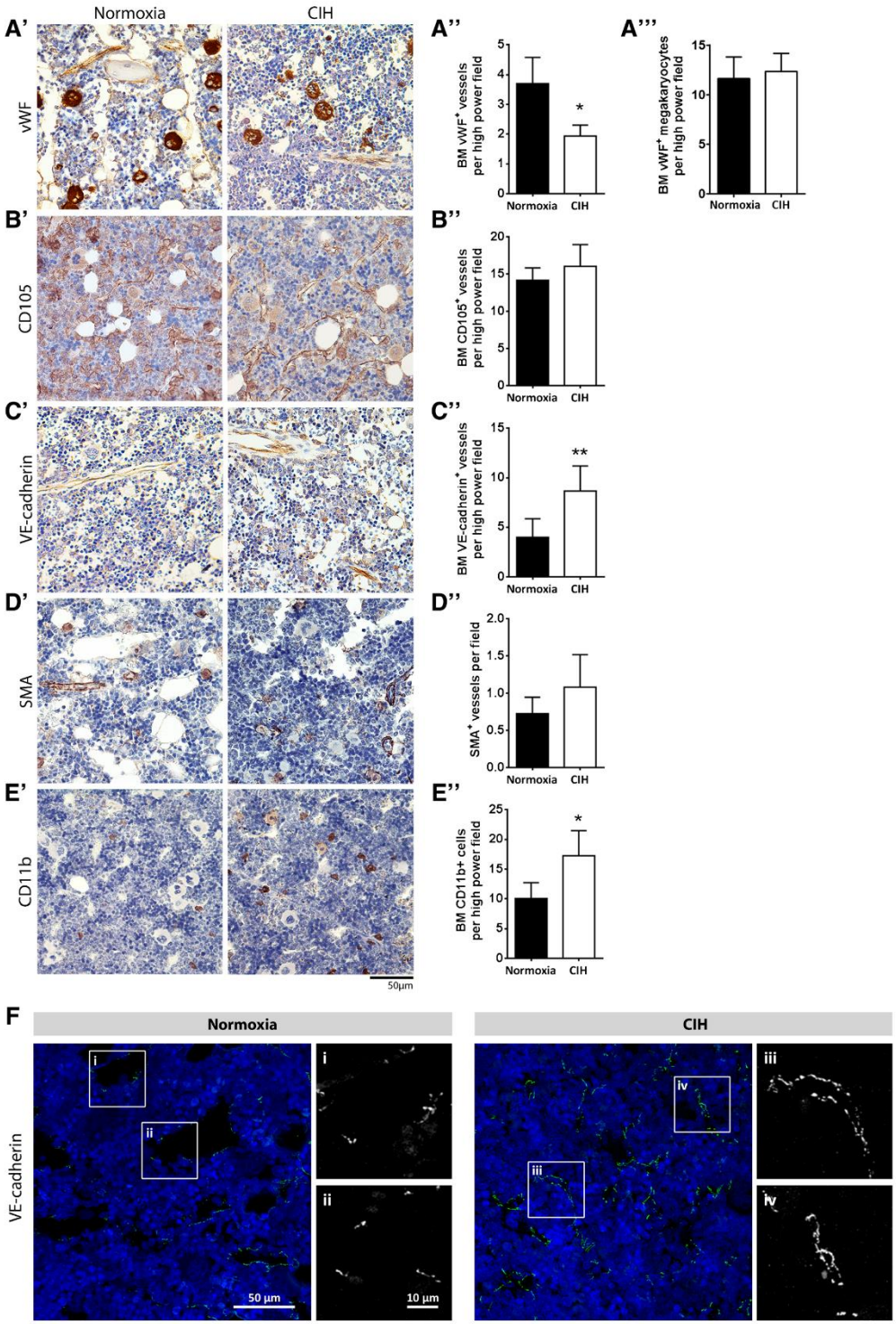


Fig. 3 Chronic intermittent hypoxia modulates circulating blood counts. **a** CIH may promote erythropoiesis. Erythrocyte, hemoglobin, and hematocrit, as well as mean corpuscular hemoglobin and mean cell hemoglobin concentration were assessed by peripheral blood cell counts. The erythrocyte count, hemoglobin, and hematocrit in CIH-exposed rats ($n = 5$) are significantly different from those in normoxia ($n = 5$) (*$p < 0.05$). **b** CIH increases circulating monocytes and decrease lymphocytes. However, peripheral blood cell counts showed no differences in neutrophils, eosinophils, or leukocytes. **c** Platelet count and mean platelet volume are not modified by exposure to CIH. Results are represented as the mean ± SD of blood samples from five male Wistar rats (*$p < 0.05$; **$p < 0.01$)

induce a smaller increase in the numbers of circulating erythrocytes [60]. However, the effects of combined CIH with hypercapnia in the BM microenvironment and hematopoiesis were not yet described. Therefore, although some data suggest that $PaCO_2$ may influence physiological responses to IH, further studies are needed to evaluate the combined effect of IH and hypercapnia. Only male rats were included in our study to avoid hypothetical effects of estrogens on hematologic responses to chronic intermittent hypoxia since it has been described that 17β-estradiol can influence the expression of hypoxia-inducible genes such as VEGF and endothelin-1 [2] and decreases hypoxic induction of erythropoietin gene expression [20, 54, 80].

In this study, we report that CIH induces a deviation from the normal body weight gain observed in normoxia-exposed rats. These results are consistent with those obtained in a parallel study, where the effects of chronic intermittent hypoxia on body weight of male Wistar rats from the NOVA Medical School colony were evaluated. We observed (unpublished

Fig. 4 Chronic intermittent hypoxia modifies the BM vascular structure. ▶ **a'–e'** Representative images of femur bone marrow stained with vWF, CD105, VE-cadherin, SMA, and CD11b counterstained with hematoxylin. **a", c", d"** BM from CIH exposed rats ($n = 6$) has more VE-cadherin$^+$ vessels and SMA coverage but less vWF$^+$ sinusoids (400×, Leica DM2500). **e', e"** Representative images of CD11b immunohistochemistry in femur BM show an increase in BM monocyte count in CIH exposed animals. (400×, Leica DM2500) **a', a"', b', b"** No changes in the total number of vessels or in megakaryocyte count were observed, as accounted by CD105 and vWF staining, respectively. Results are represented as the mean ± SD of bone marrow sections from six male Wistar rats (*$p < 0.05$; **$p < 0.01$). **f** Representative images of femur bone marrow fluorescently immunostained for VE-cadherin show an increase in total VE-cadherin vessels and in VE-cadherin vessel coverage. Scale bar, 50 µm (insets magnified 2.5×). Images were acquired with a Zeiss LSM 510 META microscope


Normoxia
CIH
A'
vWF
B'
CD105
C'
VE-cadherin
D'
SMA
E'
CD11b
50μm
A''
BM vWF+ vessels per high power field
A'''
BM vWF+ megakaryocytes per high power field
B''
BM CD105+ vessels per high power field
C''
BM VE-cadherin+ vessels per high power field
D''
SMA+ vessels per field
E''
BM CD11b+ cells per high power field
Normoxia
CIH
F
VE-cadherin
i
ii
iii
iv
50 μm
10 μm

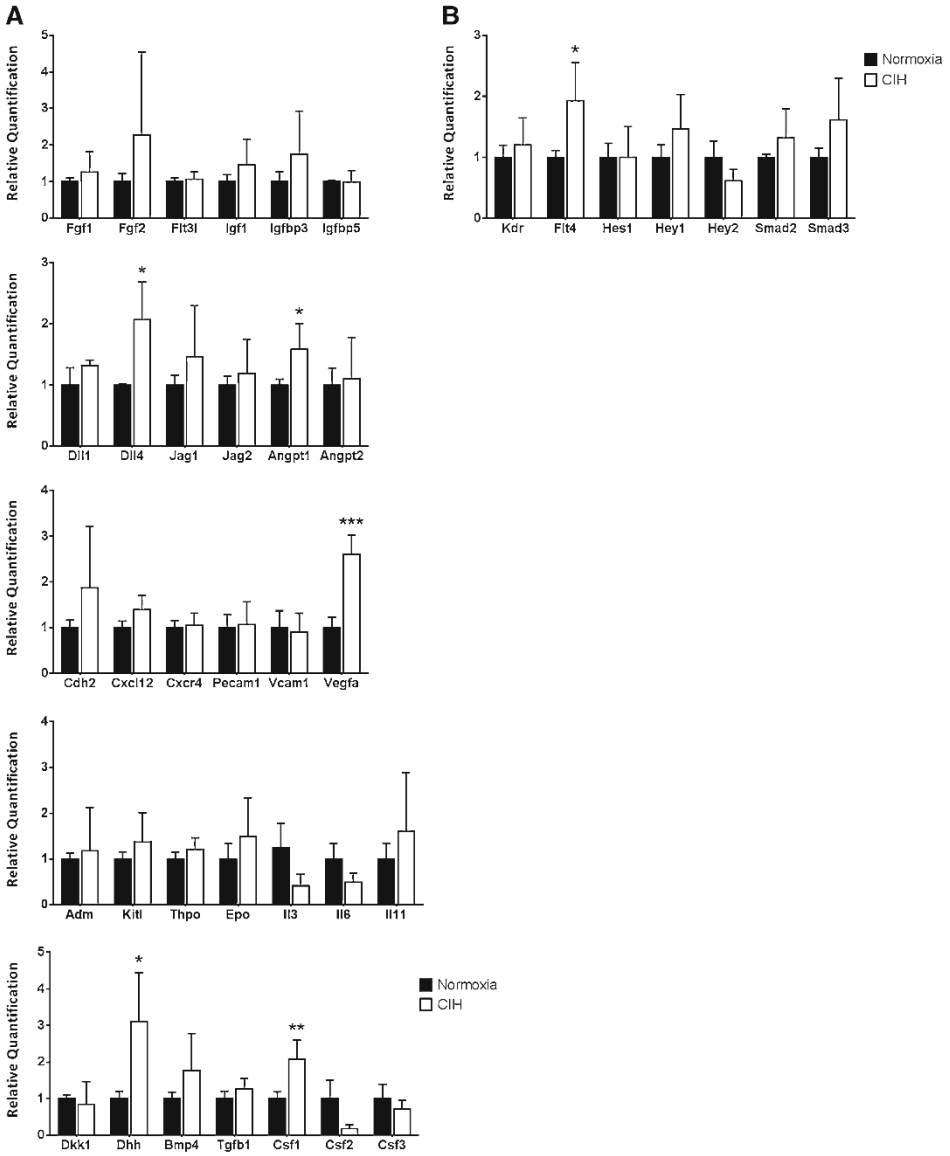


Fig. 5 Chronic intermittent hypoxia modulates bone marrow angiocrine gene expression. **a** Angiocrine gene modulation was assessed by relative quantification of mRNA of total BM samples from normoxia ($n-6$) and CIH ($n=6$) treated rats. As determined by RT-PCR, we observed an increase in *Vegfa*, *Dll4*, *Angpt1*, *Dhh*, and *Csf1*. **b** In addition, we also measured an increase in the expression of *Flt4*. Data are represented as mean ± SD of six male Wistar rats (*$p<0.05$; **$p<0.01$; ***$p<0.001$)

data) that rats exposed to 35 days of CIH weighed significantly less compared with age-matched healthy male Wistar rats kept in normoxia. Several authors reported a weight loss of CIH-exposed rats when compared with control rats [69, 70, 75, 85]. Such alteration in body weight might be explained by the production and release of leptin into the circulation as a response to hypoxia [1, 39, 51, 53, 76, 81]. Leptin is coded by an hypoxia inducible gene that acts upon the hypothalamus to control body weight by reducing food intake and increasing energy expenditure [31, 59, 81]. Moreover, leptin-deficient mice exposed to CIH have a normal weight gain, compared to normoxic mice [79], indicating that this is a specific leptin-

dependent mechanism rather than just a stress response. In our study, the lower body weight may be due to a higher metabolic activity in the hypoxic rats, as we did not observe significant differences in caloric consumption between the two groups of animals, but this assumption needs further validation.

The increased risk of CIH and OSA patients to develop cardiovascular complications led us to hypothesize that a systemic mechanism might be involved in the pathophysiology of such co-morbidities. The recruitment of bone marrow-derived cells has been amply demonstrated to be involved in the onset and progression of cardiovascular diseases [78]. The involvement of BM cells has, for instance, been shown to be important in the setting of atherosclerosis [28] and has also been implicated in hypertension [78]. Therefore, in the present study, we explored the hypothesis that CIH might affect the BM microenvironment and therefore affect hematopoiesis.

Here, we show a significant increase in BM and PB myeloid (monocyte) counts in animals exposed to CIH, accompanied by an increase in the total monocyte and granulocyte progenitor cell-derived CFUs (CFU-M and CFU-G). Several studies reported an increase in circulating granulocytes both in acute and chronic hypoxia [14, 61] and increased neutrophil and lymphocyte counts in the upper airway mucosa of OSA patients [29, 68]. Interestingly, a significantly lower percentage of macrophages was found in the mucosa of these patients [68]. Nevertheless, chronic intermittent hypoxia increases the amount of pro-inflammatory pulmonary macrophages [56]. No reports of changes in circulating monocyte or in BM myeloid counts as a response to hypoxia exposure were found. However, Roiniotis and his colleagues showed that hypoxia can have a pro-survival effect both in monocytes and macrophages [66] and Yoon described decreased myelopoiesis in *Hif1a*$^{-/-}$ embryos [82], suggesting a positive correlation between hypoxia exposure and expansion of the myeloid compartment. Our data also shows that CIH can modulate the BM lymphocyte content, a finding that had not been described either in CIH-exposed rats or OSA patients. Domagala-Kulawik and her colleagues described a decrease in circulating B cells and an increase in several T lymphocyte subsets in OSA patients, but they addressed only peripheral blood and not BM lymphocyte content [17]. Furthermore, our data is consistent with previous reports showing that stabilization of Hif1α and Hif2α in thymocytes resulted in a remarkable increase in thymocyte apoptosis [4, 13] and that HIF1α deficient chimeric mice have impaired B cell development with decreased proliferation of B cell progenitors [38].

In addition, we report an increment in circulating erythrocytes and in blood hemoglobin and hematocrit, which correlates with the higher numbers of erythroid colonies (BFU-E) derived from BM progenitors. This increase in erythroid colonies was also described in rats exposed to chronic sustained hypoxia (4 weeks). However, the authors of that study failed to detect any remarkable alterations in granulocyte-macrophage progenitor numbers [67]. Previous studies have shown that acute and chronic sustained [83] or intermittent hypoxia promoted erythropoiesis [5, 46, 49, 50, 64]. Furthermore, although there is a general lack of healthy controls in most studies, clinical data of OSA patients also suggest an increase in hemoglobin levels [12, 30] and in hematocrit [22, 32], and diurnal variations in erythropoietin levels [10] which together seem to be correlated with the severity of OSA [12, 77]. The effects of hypoxia in platelet parameters are dependent on the hypoxia administration. In detail, short-term chronic sustained hypoxia (1–4 days) was reported to promote thrombocytosis [44]. However, after 4–5 days of exposure, platelet counts returned to normal and thereafter rapidly declined between the fifth and the ninth days of hypoxia, leveling off at half their normal value [44, 48]. Studies in chronic intermittent asphyxia, however, have shown it does not affect platelet count, but instead increases platelet activation and aggregation [18], an effect that is correlated with the severity of the disease in OSA patients [35]. These results deserve further studies but emphasize the differences between sustained and intermittent hypoxia.

One interesting observation in bone marrow sections of animals in CIH was the significant increase in VE-cadherin-expressing vessels and in smooth muscle cell coverage, accompanied by a decrease in the vWF-positive vessels. However, we did not observe an increase in total vessel number assessed by CD105 expression. These findings highlight the heterogeneity of the vascular content of the BM microenvironment (similar findings, in a different context were reported in Remedio et al. 2012) [65] and demonstrate that different vascular markers should be used concomitantly, to avoid misinterpretation of single marker-staining patterns.

These morphological changes in bone marrow vessels upon CIH exposure are also indicative of a molecular process which appears to be favoring vascular stability. vWF is associated with activated and thus less stable vessels, since it is upregulated in endothelial cells treated with FGF2 and VEGF (potent angiogenic inducers) [84]. Contrastingly, VE-cadherin expression and smooth muscle coverage have been associated with increased vessel stability [21], usually induced after an active angiogenic (generating new vessels) process [55]. HIF1α, that we found to be upregulated in hypoxic rats, is one of the major inducers of angiogenesis, as it upregulates *Vegf* expression, ultimately leading to vessel permeability and instability [23, 41, 47, 71]. This process is tightly coupled with a decrease of VE-cadherin in the endothelial tight junctions [37, 47], in a VEGF-dependent manner. Our data suggest that the BM sinusoids may not be responding to the proangiogenic effects of VEGF and instead become more stable upon hypoxia exposure. Additionally, the reported increase in SMA-positive vessels in CIH-exposed rats is suggestive of vessel stabilization. This is in line with the findings that hypoxia promotes endothelial cell activation which will lead to the release of mitogenic factors for smooth muscle cells [33, 52].

An understanding of the role of vessels (and of endothelial cells that comprise them) in organ function and recovery following injury has dramatically changed in the last years. It is now accepted that endothelial cells within each organ express a different subset of trophic growth factors, known as "angiocrine factors," that will satisfy the function and metabolic demands of that specific organ. Moreover, ECs play an active role in organ recovery, through an adaptation of the expression of these trophic factors, supporting the regeneration and proliferation of stem and progenitor cells in the affected tissues, in a paracrine manner. [8, 9, 57]. In the present study, we reasoned the vascular changes seen in the bone marrows of animals exposed to CIH might affect the production of specific angiocrine factors; identification of such factors could in turn explain the alterations in hematopoiesis seen in CIH animals. We observed significant changes in the expression of *Csf1*, which explains the increase in BM and PB myeloid compartment [11], and in the levels of *Vegfa* and *Angpt1*. *Vegf* is an hypoxia-inducible gene and is most likely upregulated in response to the increased levels of *Hif1α* in hypoxic rats. Angiopoietin 1 (Angpt1) in particular has been shown to modulate vessel stability by promoting the chemoattraction of smooth muscle cells to newly formed vessels [73], usually in response to augmented VEGF levels [27]. Additionally, Angpt1 protects blood vessels from VEGF-induced permeability by inhibiting internalization of VE-cadherin which leads to an increase in VE-cadherin expression and vessel stabilization [26, 27]. Together, these molecular findings correlate with the vascular changes observed in the bone marrow of animals exposed to CIH.

Taken together, our data obtained from an animal model of OSA, reveal that the systemic effects of CIH result in modulation of the bone marrow microenvironment, namely, the bone marrow vasculature, which in turn might be perturbing hematopoiesis. Our results pave the way for pre-clinical and clinical studies aimed at validating these findings in OSA patients.

Acknowledgments The authors thank the Neo-vascularization Lab members for their input and suggestions.

This study was supported by Fundação para a Ciência e Tecnologia (FCT, Portuguese Government) grants and fellowships.

References

1. Ambrosini G, Nath AK, Sierra-Honigmann MR, Flores-Riveros J (2002) Transcriptional activation of the human leptin gene in response to hypoxia. Involvement of hypoxia-inducible factor 1. J Biol Chem 277:34601–34609. doi:10.1074/jbc.M205172200
2. Bausero P, Ben-Mahdi M-H, Mazucatelli J-P, Bloy C, Perrot-Applanat M (2000) Vascular endothelial growth factor is modulated in vascular muscle cells by estradiol, tamoxifen, and hypoxia. Am J Physiol Heart Circ Physiol 279:H2033–H2042
3. Beran M (1973) Hemopoietic recovery in posthypoxic mice: repopulation of CFUs and morphologically identifiable cells in the bone marrow and spleen. Radiat Res 53:468–479
4. Biju MP, Neumann AK, Bensinger SJ, Johnson RS, Turka LA, Haase VH (2004) Vhlh gene deletion induces Hif-1-mediated cell death in thymocytes vhlh gene deletion induces Hif-1-mediated cell death in thymocytes †. Mol Cell Biol 24:9038–9047. doi:10.1128/MCB.24.20.9038
5. Boyum A, Carsten AL, Laerum OD, Cronkite EP (1972) Kinetics of cell proliferation of murine bone marrow cells cultured in diffusion chambers: effect of hypoxia, bleeding, erythropoietin injections, polycythemia, and irradiation of the host. Blood 40:174–188
6. Bradley TR, Metcalf D (1966) The growth of mouse bone marrow cells in vitro. Aust J Exp Biol Med Sci 44:287–299
7. Briançon-Marjollet A, Pépin J-L, Weiss JW, Lévy P, Tamisier R (2014) Intermittent hypoxia upregulates serum VEGF. Sleep Med 6–7. doi: 10.1016/j.sleep.2014.07.006
8. Butler JM, Kobayashi H, Rafii S, Manuscript A (2010) Instructive role of the vascular niche in promoting tumour growth and tissue repair by angiocrine factors. Nat Rev Cancer 10:138–146. doi:10.1038/nrc2791.Instructive
9. Butler JM, Nolan DJ, Vertes EL, Varnum-finney B, Hooper AT, Seandel M, Shido K, White IA, Witte L, May C, Shawber C, Kimura Y, Kitajewski J, Rosenwaks Z, Bernstein ID, Rafii S (2010) Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. 6:251–264. doi: 10.1016/j.stem.2010.02.001.Endothelial
10. Cahan C, Decker MJ, Arnold JL, Washington LH, Veldhuis JD, Goldwasser E, Strohl KP (1992) Diurnal variations in serum erythropoietin levels in healthy subjects and sleep apnea patients. J Appl Physiol 72:2112–2117
11. Chitu V, Stanley ER (2006) Colony-stimulating factor-1 in immunity and inflammation. Curr Opin Immunol 18:39–48. doi:10.1016/j.coi.2005.11.006
12. Choi JB, Loredo JS, Norman D, Mills PJ, Ancoli-Israel S, Ziegler MG, Dimsdale JE (2006) Does obstructive sleep apnea increase hematocrit? Sleep Breath 10:155–160. doi:10.1007/s11325-006-0064-z
13. Clambey ET (2013) Hypoxia and hypoxia-inducible factors as regulators of T cell development, differentiation and function. Immunol Res 55:58–70. doi:10.1007/s12026-012-8349-8.Hypoxia
14. Coates G, Nahmias C, Thind A (1983) Platelets and leukocytes in the lungs after acute hypobaric hypoxia. J Appl Physiol 55:1536–1541
15. Coulombel L (2004) Identification of hematopoietic stem/progenitor cells: strength and drawbacks of functional assays. Oncogene 23:7210–7222. doi:10.1038/sj.onc.1207941
16. Diogo LN, Monteiro EC (2014) The efficacy of antihypertensive drugs in chronic intermittent hypoxia conditions. Front Physiol 5:361. doi:10.3389/fphys.2014.00361
17. Domagala-Kulawik J, Osinska I, Piechuta A, Bielicki P, Skirecki T (2015) T, B, and NKT cells in systemic inflammation in obstructive sleep apnoea. Mediat Inflamm
18. Dunleavy M, Dooley M, Cox D, Bradford A (2005) Chronic intermittent asphyxia increases platelet reactivity in rats. Exp Physiol 90:411–416. doi:10.1113/expphysiol.2004.029306
19. Dyavanapalli J, Jameson H, Dergacheva O, Jain V, Alhusayyen M, Mendelowitz D (2014) Chronic intermittent hypoxia-hypercapnia blunts heart rate responses and alters neurotransmission to cardiac vagal neurons. J Physiol 592:2799–2811. doi:10.1113/jphysiol.2014.273482

20. Ebert BL, Bunn HF (1999) Regulation of the erythropoietin gene. Blood 94:1864–1877. doi:10.1097/00062752-199502020-00004
21. Eisa-Beygi S, Macdonald RL, Wen X-Y (2014) Regulatory pathways affecting vascular stabilization via VE-cadherin dynamics: insights from zebrafish (Danio rerio). J Cereb Blood Flow Metab 34:1430–1433. doi:10.1038/jcbfm.2014.128
22. Eisensehr I, Noachtar S (2001) Haematological aspects of obstructive sleep apnoea. Sleep Med Rev 5:207–221. doi:10.1053/smrv.2001.0158
23. Engelhardt S, Al-Ahmad AJ, Gassmann M, Ogunshola OO (2014) Hypoxia selectively disrupts brain microvascular endothelial tight junction complexes through a hypoxia-inducible factor-1 (HIF-1) dependent mechanism. J Cell Physiol 229:1096–1105. doi:10.1002/jcp.24544
24. Epstein LJ, Jervis OJ, Henderson JH, Sullivan M, Mohsenifar Z (2001) Measurement of gastric P(CO2) as an index of tissue hypoxia during obstructive sleep apnea. Respiration 68:28–34. doi:50459
25. Fletcher EC, Lesske J, Qian W, Miller CC, Unger T (1992) Repetitive, episodic hypoxia causes diurnal elevation of blood pressure in rats. Hypertension 19:555–561. doi:10.1161/01.HYP.19.6.555
26. Gavard J (2009) Breaking the VE-cadherin bonds. FEBS Lett 583:1–6. doi:10.1016/j.febslet.2008.11.032
27. Gavard J, Patel V, Gutkind JS (2008) Angiopoietin-1 prevents VEGF-induced endothelial permeability by sequestering Src through mDia. Dev Cell 14:25–36. doi:10.1016/j.devcel.2007.10.019
28. Ghattas A, Griffiths HR, Devitt A, Lip GYH, Shantsila E (2013) Monocytes in coronary artery disease and atherosclerosis: where are we now? J Am Coll Cardiol 62:1541–1551. doi:10.1016/j.jacc.2013.07.043
29. Hauber H-P, Rüller S, Müller E, Hansen E, Zabel P (2011) Pharyngeal lavage lymphocytosis in patients with obstructive sleep apnea: a preliminary observation. PLoS One 6, e16277. doi:10.1371/journal.pone.0016277
30. Heinicke K, Prommer N, Cajigal J, Viola T, Behn C, Schmidt W (2003) Long-term exposure to intermittent hypoxia results in increased hemoglobin mass, reduced plasma volume, and elevated erythropoietin plasma levels in man. Eur J Appl Physiol 88:535–543. doi:10.1007/s00421-002-0732-z
31. Hill JW, Williams KW, Ye C, Luo J, Balthasar N, Coppari R, Cowley MA, Cantley LC, Lowell BB, Elmquist JK (2008) Acute effects of leptin require PI3K signaling in hypothalamic proopiomelanocortin neurons in mice. J Clin Invest 118:1796–1805. doi:10.1172/JCI32964
32. Hoffstein V, Herridge M, Mateika S, Redline S, Strohl KP (1994) Hematocrit levels in sleep apnea. Chest 106:787–791
33. Humar R, Kiefer FN, Berns H, Resink TJ, Battegay EJ (2002) Hypoxia enhances vascular cell proliferation and angiogenesis in vitro via rapamycin (mTOR)-dependent signaling. FASEB J 16:771–780. doi:10.1096/fj.01-0658com
34. Imanirad P, Dzierzak E (2013) Hypoxia and HIFs in regulating the development of the hematopoietic system. Blood Cells Mol Dis 51:256–263. doi:10.1016/j.bcmd.2013.08.005
35. Kanbay A, Tutar N, Kaya E, Buyukoglan H, Ozdogan N, Oymak FS, Gulmez I, Demir R (2013) Mean platelet volume in patients with obstructive sleep apnea syndrome and its relationship with cardiovascular diseases. Blood Coagul Fibrinolysis 24:532–536. doi:10.1097/MBC.0b013e32835e98da
36. Kantores C, McNamara PJ, Teixeira L, Engelberts D, Murthy P, Kavanagh BP, Jankov RP (2006) Therapeutic hypercapnia prevents chronic hypoxia-induced pulmonary hypertension in the newborn rat. Am J Physiol Lung Cell Mol Physiol 291:L912–L922. doi:10.1152/ajplung.00480.2005
37. Kobayashi S, Yamashita T, Ohneda K, Nagano M, Kimura K, Nakai H, Poellinger L, Ohneda O (2015) Hypoxia-inducible factor-3α promotes angiogenic activity of pulmonary endothelial cells by repressing the expression of the VE-cadherin gene. Genes Cells. doi:10.1111/gtc.12215
38. Kojima H, Gu H, Nomura S, Caldwell CC, Kobata T, Carmeliet P, Semenza GL, Sitkovsky MV (2002) Abnormal B lymphocyte development and autoimmunity in hypoxia-inducible factor 1alpha -deficient chimeric mice. Proc Natl Acad Sci U S A 99:2170–2174. doi:10.1073/pnas.052706699
39. Koob GF, Annau Z, Rubin RJ, Montgomery MR, Robert J (1974) Effect of hypoxic hypoxia and carbon monoxide on food intake, water intake, and body weight in two strains of rats. Life Sci 14:1511–1520. doi:10.1016/0024-3205(74)90162-3
40. Kopp H-G, Avecilla ST, Hooper AT, Rafii S (2005) The bone marrow vascular niche: home of HSC differentiation and mobilization. Physiology 20:349–356. doi:10.1152/physiol.00025.2005
41. Koto T, Takubo K, Ishida S, Shinoda H, Inoue M, Tsubota K, Okada Y, Ikeda E (2007) Hypoxia disrupts the barrier function of neural blood vessels through changes in the expression of claudin-5 in endothelial cells. Am J Pathol 170:1389–1397. doi:10.2353/ajpath.2007.060693
42. Krieger J, Sforza E, Barthelmebs M, Imbs J-L, Kurtz D (1990) Overnight decrease in hematocrit after nasal CPAP treatment in patients with OSA. Chest 97:729–730
43. Lavie L, Kraiczi H, Hefetz A, Ghandour H, Perelman A, Hedner J, Lavie P (2002) Plasma vascular endothelial growth factor in sleep apnea syndrome: effects of nasal continuous positive air pressure treatment. Am J Respir Crit Care Med 165:1624–1628. doi:10.1164/rccm.20110-040OC
44. Lebedeva EV, Yushkov BG, Chereshnev VA (2003) Megakaryocytopoiesis under hypoxic conditions. Bull Exp Biol Med 136:554–556
45. Lippl FJ, Neubauer S, Schipfer S, Lichter N, Tufman A, Otto B, Fischer R (2010) Hypobaric hypoxia causes body weight reduction in obese subjects. Obesity (Silver Spring) 18:675–681. doi:10.1038/oby.2009.509
46. Lord BIL, Murphy MJ (1973) Hematopoietic stem cell regulation.I. Acute effects of hypoxic-hypoxia on CFU kinetics. Blood 42:81–87
47. Makarenko VV, Usatyuk PV, Yuan G, Lee MM, Nanduri J, Natarajan V, Kumar GK, Prabhakar NR (2014) Intermittent hypoxia-induced endothelial barrier dysfunction requires ROS-dependent MAP kinase activation. Am J Physiol Cell Physiol 60637:745–752. doi:10.1152/ajpcell.00313.2013
48. McDonald TP, Cottrell M, Clift R (1978) Effects of short-term hypoxia on platelet counts of mice. Blood 51:165–175
49. McGuire M, Bradford A (1999) Chronic intermittent hypoxia increases haematocrit and causes right ventricular hypertrophy in the rat. Respir Physiol 117:53–58
50. McGuire M, Bradford A (2001) Chronic intermittent hypercapnic hypoxia increases pulmonary arterial pressure and haematocrit in rats. Eur Respir J 18:279–285
51. Messenger SA, Ciriello J (2013) Effects of intermittent hypoxia on leptin signalling in the carotid body. Neuroscience 232:216–225
52. Michiels C, Arnould T, Remacle J (2000) Endothelial cell responses to hypoxia: initiation of a cascade of cellular interactions. Biochim Biophys Acta Mol Cell Res 1497:1–10. doi:10.1016/S0167-4889(00)00041-0
53. Moreau JM, Ciriello J (2013) Effects of acute intermittent hypoxia on energy balance and hypothalamic feeding pathways. Neuroscience 253:350–360. doi:10.1016/j.neuroscience.2013.09.007
54. Mukundan H, Resta TC, Kanagy NL (2002) 17Beta-estradiol decreases hypoxic induction of erythropoietin gene expression. Am J

Physiol Regul Integr Comp Physiol 283:R496–R504. doi:10.1152/ajpregu.00573.2001
55. Murakami M (2012) Signaling required for blood vessel maintenance: molecular basis and pathological manifestations. Int J Vasc Med 2012:293641. doi:10.1155/2012/293641
56. Nagai H, Kuwahira I, Schwenke DO, Tsuchimochi H, Nara A, Ogura S, Sonobe T, Inagaki T, Fujii Y, Yamaguchi R, Wingenfeld L, Umetani K, Shimosawa T, Yoshida K, Uemura K, Pearson JT, Shirai M (2015) Pulmonary macrophages attenuate hypoxic pulmonary vasoconstriction via β3AR/iNOS pathway in rats exposed to chronic intermittent hypoxia. PLoS One 10, e0131923. doi:10.1371/journal.pone.0131923
57. Nolan DJ, Ginsberg M, Israely E, Palikuqi B, Poulos MG, James D, Ding B, Schachterle W, Liu Y, Rosenwaks Z, Jason M, Xiang J, Rafii A, Shido K, Rabbany SY, Elemento O (2014) Molecular signatures of tissue-specific microvascular endothelial cell heterogeneity in organ maintenance and regeneration. Dev Cell. doi:10.1016/j.devcel.2013.06.017.Molecular
58. Ooi H, Cadogan E, Sweeney M, Howell K, O'Regan RG, McLoughlin P (2000) Chronic hypercapnia inhibits hypoxic pulmonary vascular remodeling. Am J Physiol Heart Circ Physiol 278:H331–H338
59. Palmer BF, Clegg DJ (2013) Ascent to altitude as a weight loss method: the good and bad of hypoxia inducible factor activation. Obesity (Silver Spring). doi:10.1002/oby.20499
60. Pepelko WE (1971) Effect of hypoxia and hypercapnia alone and in combination upon the circulating red cell volume of rats. Exp Biol Med 136:967–971. doi:10.3181/00379727-136-35407
61. Petursson SR, Chervenick PA (1987) Effects of hypoxia on megakaryocytopoiesis and granulopoiesis. Eur J Haematol 39:267–273. doi:10.1111/j.1600-0609.1987.tb00768.x
62. Pugh CW, Ratcliffe PJ (2003) Regulation of angiogenesis by hypoxia: role of the HIF system. Nat Med 9:677–684. doi:10.1038/nm0603-677
63. Rathinam C, Poueymirou WT, Rojas J, Murphy AJ, Valenzuela DM, Yancopoulos GD, Rongvaux A, Eynon EE, Manz MG, Flavell RA (2011) Efficient differentiation and function of human macrophages in humanized CSF-1 mice. Blood 118:3119–3128. doi:10.1182/blood-2010-12-326926
64. Reissmann KR (1950) Studies on the mechanism of erythropoietic stimulation in parabiotic rats during hypoxia. Blood 5:372–380
65. Remédio L, Carvalho T, Caiado F, Bastos-Carvalho A, Martins D, Duarte A, Yagita H, Dias S (2012) Context- and cell-dependent effects of Delta-like 4 targeting in the bone marrow microenvironment. PLoS One 7, e52450. doi:10.1371/journal.pone.0052450
66. Roiniotis J, Dinh H, Masendycz P, Turner A, Elsegood CL, Scholz GM, Hamilton JA (2009) Hypoxia prolongs monocyte/macrophage survival and enhanced glycolysis is associated with their maturation under aerobic conditions. J Immunol 182:7974–7981. doi:10.4049/jimmunol.0804216
67. Rolović Z, Basara N, Biljanović-Paunović L, Stojanović N, Suvajdzić N, Pavlović-Kentera V (1990) Megakaryocytopoiesis in experimentally induced chronic normobaric hypoxia. Exp Hematol 18:190–194
68. Salerno F, Carpagnano E, Guido P, Bonsignore M, Roberti A, Aliani M, Vignola A, Spanevello A (2004) Airway inflammation in patients affected by obstructive sleep apnea syndrome. Respir Med 98:25–28
69. Silva AQ, Schreihofer AM (2011) Altered sympathetic reflexes and vascular reactivity in rats after exposure to chronic intermittent hypoxia. J Physiol 589:1463–1476. doi:10.1113/jphysiol.2010.200691
70. Soukhova-O'Hare GK, Ortines RV, Gu Y, Nozdrachev AD, Prabhu SD, Gozal D (2008) Postnatal intermittent hypoxia and developmental programming of hypertension in spontaneously hypertensive rats: the role of reactive oxygen species and L-Ca2+ channels. Hypertension 52:156–162. doi:10.1161/HYPERTENSIONAHA.108.110296
71. Stenmark KR, Fagan KA, Frid MG (2006) Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. Circ Res 99:675–691. doi:10.1161/01.RES.0000243584.45145.3f
72. Streeter RG, Pepelko WE, Cain SM (1975) Tissue oxygenation and splenic erythropoiesis during chronic hypoxia and hypercapnia. J Appl Physiol 38:309–314
73. Sundberg C, Kowanetz M, Brown LF, Detmar M, Dvorak HF (2002) Stable expression of angiopoietin-1 and other markers by cultured pericytes: phenotypic similarities to a subpopulation of cells in maturing vessels during later stages of angiogenesis in vivo. Lab Investig 82:387–401
74. Tilkian AG, Guilleminault C, Schroeder JS, Lehrman KL, Simmons FB, Dement WC (1976) Hemodynamics in sleep-induced apnea. Studies during wakefulness and sleep. Ann Intern Med 85:714–719
75. Totoson P, Fhayli W, Faury G, Korichneva I, Cachot S, Baldazza M, Ribuot C, Pépin J-L, Lévy P, Joyeux-Faure M (2013) Atorvastatin protects against deleterious cardiovascular consequences induced by chronic intermittent hypoxia. Exp Biol Med (Maywood) 238:223–232. doi:10.1177/1535370212473696
76. Tschöp M, Strasburger CJ, Hartmann G, Biollaz J, Bärtsch P (1998) Raised leptin concentrations at high altitude associated with loss of appetite. Lancet 352:1119–1120. doi:10.1016/S0140-6736(05)79760-9
77. Winnicki M, Shamsuzzaman A, Lanfranchi P, Accurso V, Olson E, Davison D, Somers VK (2004) Erythropoietin and obstructive sleep apnea. Am J Hypertens 17:783–786. doi:10.1016/j.amjhyper.2004.04.011
78. Xu J-M, Shi G-P (2012) Emerging role of mast cells and macrophages in cardiovascular and metabolic diseases. Endocr Rev 33:71–108. doi:10.1210/er.2011-0013
79. Yang R, Sikka G, Larson J, Watts VL, Niu X, Ellis CL, Miller KL, Camara A, Reinke C, Savransky V, Polotsky VY, O'Donnell CP, Berkowitz DE, Barouch LA (2011) Restoring leptin signaling reduces hyperlipidemia and improves vascular stiffness induced by chronic intermittent hypoxia. Am J Physiol Heart Circ Physiol 300:H1467–H1476. doi:10.1152/ajpheart.00604.2009
80. Yeo EJ, Cho YS, Kim MS, Park JW (2008) Contribution of HIF-1a or HIF-2a to erythropoietin expression: in vivo evidence based on chromatin immunoprecipitation. Ann Hematol 87:11–17. doi:10.1007/s00277-007-0359-6
81. Yingzhong Y, Droma Y, Rili G, Kubo K (2006) Regulation of body weight by leptin, with special reference to hypoxia-induced regulation. Intern Med 45:941–946. doi:10.2169/internalmedicine.45.1733
82. Yoon D, Pastore YD, Divoky V, Liu E, Mlodnicka AE, Rainey K, Ponka P, Semenza GL, Schumacher A, Prchal JT (2006) Hypoxia-inducible factor-1 deficiency results in dysregulated erythropoiesis signaling and iron homeostasis in mouse development. J Biol Chem 281:25703–25711. doi:10.1074/jbc.M602329200
83. Yoon D, Ponka P, Prchal JT (2011) Hypoxia. 5. Hypoxia and hematopoiesis. Am J Physiol Cell Physiol 300:C1215–C1222. doi:10.1152/ajpcell.00044.2011
84. Zanetta L, Marcus SG, Vasile J, Dobryansky M, Cohen H, Eng K, Shamamian P, Mignatti P (2000) Expression of Von Willebrand factor, an endothelial cell marker, is up-regulated by angiogenesis factors: a potential method for objective assessment of tumor angiogenesis. Int J Cancer 85:281–288
85. Zoccal DB, Simms AE, Bonagamba LGH, Braga VA, Pickering AE, Paton JFR, Machado BH (2008) Increased sympathetic outflow in juvenile rats submitted to chronic intermittent hypoxia correlates with enhanced expiratory activity. J Physiol 586:3253–3265. doi:10.1113/jphysiol.2008.154187